DEVELOPMENT OF MICRONEEDLE PATCHES FOR MEASLES-RUBELLA VACCINATION AND EXTENDED DELIVERY VACCINATION

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DEVELOPMENT OF MICRONEEDLE PATCHES FOR MEASLES-RUBELLA VACCINATION AND EXTENDED DELIVERY VACCINATION

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To my family for their never-ending support

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ASC	Antibody Secreting Cells
BSA	Bovine Serum Albumin
CAN-BD	Carbon dioxide Assisted Nebulization with a Bubble Dryer
CDC	Centers for Disease Control and Prevention
CMC	Carboxymethylcellulose
CRS	Congenital Rubella Syndrome
DMEM	Dulbecco Modified Eagle Medium
DU	D-antigen Unit
FBS	Fetal Bovine Serum
GC B	Germinal Center B cells
HAI	Hemagglutination Inhibition
HCW	Health Care Worker
IACUC	Institutional Animal Care and Use Committee
ID	Intradermal
IPV	Inactivated Polio Vaccine
MiG	Measles Immunoglobin
MMR	Measles Mumps and Rubella
MN	Microneedle
MNP	Microneedle Patch
MR	Measles and Rubella
MV-GFP	Measles Virus- Green Fluorescent Protein
N	Nucleocapsid

OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cell
PDMS	Polydimethylsiloxane
PLGA	Poly(lactic-co-glycolic) Acid
PVA	Poly(vinyl alcohol)
$TCID_{50}$	Tissue Culture Infectivity Dose 50%
Tfh	T Follicular Helper cells
VVM	Vaccine Vial Monitor
WHO	World Health Organization

SUMMARY

Despite cheap and effective vaccines, nearly 1.5 million children die each year from vaccine preventable diseases. The World Health Organization has called for novel vaccine technologies that can reduce needle-and-syringe use, reduce the dose of vaccine required, and reduce vaccine wastage, all while inducing an appropriate immune response. Microneedle patches offer a unique method of delivering vaccines into the skin without the use of needles and syringes. These patches consist of an array of needles in which each needle is composed of water-soluble polymers, sugars, excipients, and the vaccine. When inserted into the skin, the needles dissolve and deliver their embedded cargo. These patches meet a critical need for vaccination campaigns. They come in small, single dose packaging and can be administered by minimally trained personnel. Once dissolved, the needles leave no sharps waste. Additionally, the patches can be stored at elevated temperatures without damage to the vaccine. When the vaccine is delivered into the skin, studies have shown improved immune response compared to a standard intramuscular injection.

This work developed two novel uses for microneedle patches. The first innovation delivers both measles and rubella vaccines in a single patch. Formulations were developed to minimize the loss of vaccine activity during the manufacturing process and subsequent storage at elevated temperatures. These patches were shown to be immunogenic in juvenile and infant rhesus macaques, and vaccination with a microneedle patch was able to protect the infants from a live measles viral challenge. The second project investigated a novel vaccination strategy using daily dosing to improve the immune response compared to a bolus injection. In this work, daily intradermal injections or daily microneedle patches were

used to deliver vaccines over the course of one month, and this regime induced responses comparable to two bolus doses. The microneedle technologies developed in this work can improve vaccine coverage.

CHAPTER 1. INTRODUCTION

1.1 Vaccination Campaigns

Vaccines are acclaimed as one of the top ten public health interventions of the past century [1]. They have been influential in the eradication of smallpox, near eradication of polio, and significant reduction of disease [2]. However, over 19 million children do not receive the basic vaccinations recommended by the World Health Organization (WHO), resulting in approximately 1.5 million children deaths from vaccine preventable diseases each year [3]. While the number of vaccines administered has been rising, the Midterm Global Vaccine Action Plan in 2016 notes that vaccination coverage rates are stagnant and recommends the acceleration of novel vaccination technologies to improve global access to vaccination [2]. Currently, almost all recommended vaccinations occur using a needle and syringe to deliver the vaccine to the muscle or subcutaneous area. This technology has many setbacks, especially in developing countries with a common lack of infrastructure and resources. New vaccination implementations should be small and easy to transport, thermostable, easy to administered, and immunogenic. Thus, there is a significant need for technologies that can easy logistics and reduce the costs of vaccinations.

1.2 Microneedle Patches

1.2.1 Dissolving Microneedle Patches

Microneedle (MN) patches are a novel delivery technology to deliver drugs and vaccines into the skin and have been under development for the past twenty years [4]. These needles can be divided into four categories: (1) solid MNs which pierce the skin

followed by administration of the drug in liquid or patch form; (2) hollow MNs for delivery of a liquid drug solution; (3) metal MNs coated with a dried drug film; and (4) dissolving polymeric MN in which the drug is loaded in the polymer needle tip [5]. For the purpose of this work, only dissolving polymeric MN patches will be discussed further.

MN patches consist of an array of polymeric MNs. Generally, this ~1 cm^2 array consists of a 10x10 grid of needles, each several hundred microns tall, on a larger rigid backing [6-10]. The needles are composed of a variety of biocompatible, water-soluble materials such as sugars, carboxymethylcellulose (CMC), polyvinyl alcohol (PVA), polyvinylpyrrolidone, and chitosan [4, 9, 11-14]. These materials encase the drug or vaccine in the tip of the MN. The MNs are mechanically strong enough to painlessly pierce the upper layers of the skin [6, 14-16]. The moisture in the skin dissolves the polymers and sugars and releases the cargo. After insertion, the backing material can be discarded as non-sharp biomedical waste [16]. These patches have been used to deliver a variety of therapeutic molecules, such as DNA [17], insulin [8], small molecule therapeutics [18], and vaccines [9, 13, 16, 19, 20].

1.2.2 Microneedle Patches for Vaccinations

MN patches over numerous advantages over the traditional needle and syringe delivery technology in clinics in developed countries as well as rural vaccination campaigns. First, MN patches are packaged in small, single-use, single-dose units. This can increase the safety of vaccination while decreasing vaccine waste. For example, multi-dose vials of vaccines are often used in vaccination campaigns leading to vaccine wastage if not all doses are used [21]. If only a few more patients are present at the end of a

vaccination session, health care workers must decide between (1) opening a new vial for a few children or (2) saving the vaccine but risking under vaccinating the community [21].

Currently, vaccines are shipped through the cold chain in a lyophilized or liquid state. The cold chain is a sequence of refrigerators or freezers that maintain cold temperatures, typically 2-8˚C or -20˚C, respectively, from the manufacturing site, through regional distribution points, up to the vaccination site [22]. The cold chain is extremely expensive, costing $200-$300 million per year, and breaks in the chain can lead to vaccine wastage or administration of less-effective vaccines [23-26]. In the case of MN patches, the vaccine is entrapped in a dried state. Due to the immobility of the proteins, vaccine activity can be retained for longer time period at elevated temperatures [9, 26-29]. However, each vaccine requires a novel formulation which can retain vaccine activity during MN manufacturing and storage. These studies can be time and resource intensive [30].

Prior to vaccination, lyophilized vaccines must be reconstituted with sterile liquid, mixed, and properly aliquoted. Reconstitution leads to potential mistakes, increases costs of vaccination campaigns, and requires staff training [31, 32]. Administration of MN patches is simple. No external paraphernalia such as reconstitution vials, sharps containers, needles, or syringes are required, thus reducing the weight that must be transported to and from vaccination sites [32]. Very little preparatory work is required for patch insertion. As compared to the Mantoux method which requires hours of training, MN patches can be administered via minimally trained personnel or even self-administered [16, 33]. This would be more cost-effective in vaccination campaigns or allow for in-home delivery of vaccinations [9, 34]. Once the patches are administered, the needles are fully dissolved and

can be discarded as biomedical waste, as a used bandage would be [5]. This eliminates the possibility of re-stick injuries or needle re-use. Over one million deaths are caused each year due to re-stick injuries [35-37]; in 2017, 15 children died in Sudan due to needle re-use during a measles vaccination campaign [38].

Most vaccines require multiple doses to invoke a strong response in all patients [39]. MN patches can induce strong immune response to a vaccination and in some cases outperform other vaccination locations. MN patches deliver antigen into the skin, an organ rich in antigen-presenting cells such as epidermal Langerhans cells and dermal dendritic cells [40]. These cells are adept at encountering and responding to pathogens in the skin; effective lymphatic drainage can increase the immune system's response. Due to the enhanced immune response, dose sparing (i.e. using a reduced dose to induce an equivalent response) has been demonstrated for some vaccines in animal models [41-44]. Additionally, MN patches have been shown to be more effective than IM injections in young mice [45]. Clinical trials have demonstrated that vaccination with MN patches can translate into humans [16].

1.3 Measles and Rubella

1.3.1 Disease Burden

Measles virus remains one of the leading causes of vaccine-preventable deaths [46]. Measles infection is associated with a rash, fever, cough, and immune suppression [46, 47]. Respiratory tract infections and pneumonia are the most common measles-associated deaths [46]. Measles is extremely infectious and spreads through aerosolized particles [47, 48]. Due to the high infectivity rate, high herd immunity over 95% is

necessary to block disease transmission [46]. Before the introduction of the vaccine, over 2 million deaths per year were attributed to measles [47]; in 2014, that number has fallen to 115,000 children each year [49]. In 2000, measles deaths accounted for 5% of all under-five mortality [50]. Measles was eliminated from the US in 2000 [48]; however, it is making a comeback in small pockets of unvaccinated residents or in unvaccinated travelers [46, 48].

Rubella is typically a much milder disease than measles. Older children and adults usually make a fully recovery after infection [49]. However, when pregnant women are infected, the infection can result in miscarriage or congenital rubella syndrome (CRS) [51]. CRS leads to disabling conditions affecting the heart, brain, eyes, and ears; over 100,000 children are born each day with CRS [49]. A single case of CRS can cost over $75,000 over a lifetime [52]. Rubella was eliminated from the Americas in 2015; however, global vaccination coverage remains low at 46% [49].

1.3.2 Eradication Efforts

In 2010, the WHO determined that measles can and should be eradicated [53]. In the following years, both the Measles and Rubella (MR) Initiative, supported by American Red Cross, CDC, the United Nations Foundation, UNICEF and WHO, and the Global Vaccine Action Plan have set out elimination goals. The initial goals were to eliminate measles in 5 WHO regions by 2020 [53]. The general strategy was to provide the first dose of measles vaccine via routine health services and the second dose of measles vaccine through mass vaccination campaigns. Through increased vaccination efforts, coverage rose to approximately 85% for the first dose of measles vaccine, 61% for the second dose, and

46% for the first dose of rubella vaccine [49]. From 2012-2014, 4.25 million measles deaths were averted [49]. However, vaccinations rates have stagnated, and an estimated 21 million infants did not receive their first dose of measles vaccines in 2008 [53]. This progress is insufficient to reach the 2020 elimination goals. Implementing novel vaccine technologies, building on the polio eradication efforts, and strengthening surveillance systems will be crucial in attaining the next phase of vaccination and elimination goals [53].

1.3.3 Vaccines

MR vaccines are extremely effective vaccines and have an estimated annual net benefit of over $9.7 billion dollars each year [54]. A dose between 10^3 and 10^4 tissue culture infectivity units ($TCID_{50}$) of each of the live-attenuated vaccines is administered subcutaneously in 0.5 mL using a needle and syringe [55]. Measles, mumps, and rubella (MMR) vaccines were combined in 1971 [47]. The most common strains are Edmonstron-Zagreb measles virus and Wistar RA 27/3 rubella virus [46, 55]. The lyophilized vaccines can be stored for at least two years at 2-8˚C, eight months at 22˚C, and one month at 37˚C [55]. Reconstituted vaccine is far less stable with 50% loss in one hour at 25˚C and almost 100% loss in one hour at 37˚C. Additionally, the vaccine is sensitive to sunlight [55]. The vaccination invokes both humoral and cellular immune responses. IgM is present between two and six weeks after vaccination in serum, while IgG persists for many years [56]. IgA is found mostly in nasal secretions [56]. After a single dose, 95% of 12 month old children are protected from measles; vaccination at this age provides many years of protection, potentially lifelong protection [46, 47]. Vaccination can be boosted by vaccination or wild-type exposure, with 99% of children seropositive after a second dose [46].

1.3.4 Alternative Delivery Methods

To overcome the limitations of the current lyophilized, injected vaccines, other delivery methods have been developed and tested in animal models or the clinic with mixed success. Skin vaccination is a popular method for many of the reasons presented above. Transcutaneous immunizations were performed by tape stripping to remove the stratum corneum followed by liquid vaccine underneath a bandage. While being more acceptable by patients, the immune response was inferior to subcutaneous injection, probably due to incomplete delivery [57]. Jet injectors which use high-pressured stream of liquid to push through the upper layers of the skin have been used with more success. In clinical studies with MMR, this delivery method was well tolerated by patients and had minimal adverse events (i.e. site soreness, malaise, fever). In an adolescent (10-14 years) study, the jet injector performed equivalently to a needle and syringe [58]. Non-inferiority to needle and syringe delivery was demonstrated in toddlers (9-23 month); jet injector and needle and syringe outperformed bifurcated needle and nose drops delivering the same vaccine [59]. However, in infants, the response to rubella was non-inferior to needle and syringe delivery [60]. The measles and mumps seroconversion rates were inferior to the needle and syringe delivery. Study nurses noted skin wetness after some vaccinations using the jet injector, which could indicate that incomplete delivery is the cause of the lowered immune response [60].

Aerosol vaccination can ease logistics of needle and syringe deliver and may better protect against measles viruses by vaccinating at the site of infection. In non-human primates, carbon dioxide assisted nebulization with a bubble-dryer (CAN-BD) vaccination offered complete protection from measles virus challenge [61]. However, intra-tracheal

delivery of dry powders produced an inferior response compared to an injection or nebulized aerosol [62, 63]. Clinical trials have produced conflicting results. First, in a study in children ages 5-14, aerosolized vaccines induced no serious side effects and had a similar immune response to subcutaneous injection [64]. In two adult studies, no safety issues were noted. The aerosol vaccination induced an immune response comparable to subcutaneous injection, but the differences between the two routes is hard to parse because all subjects were measles positive at time of vaccination [65, 66]. Finally, the only study delivering the first dose of measles vaccine with infant patients demonstrated that aerosolized vaccination was inferior to subcutaneous injection [67].

1.3.5 Vaccine Stability

Live-attenuated vaccines must retain their ability to infect host cells and replicate in order to induce a potent immune response. Inactivated measles vaccine was licensed in the early 1960s; however, this was discontinued after cases of atypical measles appeared. When vaccinated children became infected with measles virus, they experienced high prolonged fever, pulmonary nodules, extensive rash, and enlarged lymph nodes, indicating the need to deliver live viral particles not inactivated ones [68]. In order to meet the demands of vaccination campaigns, the WHO requires that novel MR vaccine technologies cannot lose more than one log of activity after one week at 37˚C [69]. The activity of these vaccines is measured via cellular assays, as previously described [70, 71].

1.3.5.1 Dried state

MR vaccines must maintain activity during MN patch manufacturing in which the vaccine is dried while under vacuum and during storage in which the patches are shipped

and may experience elevated temperatures. Lyophilized vaccines are more stable than in liquid state due to the immobilization of the proteins [72]. Two other methods have been developed to stabilize measles vaccine. First, CAN-BD creates powders of vaccine and excipients [73]. This method can lower the water content to 0.5%, compared with 1% in the lyophilized vaccine containing sorbitol. Low water content is important to maintain vaccine activity and retain powder integrity [73]. Trehalose and sorbitol maintained 60% of the original activity during drying, but myo-inositol was necessary to reduce loss during storage.

Spray drying is gaining popularity due to its high-throughput, flexible manufacturing process. When spray drying measles vaccine, a mixture of excipients of sucrose, trehalose, arginine, human serum albumin, and glycerol in potassium phosphate buffer was necessary to maintain activity [74]. Divalent cations aided the long-term stability of the resulting powders. These powders lost one log of activity after 8 weeks at 37˚C; during storage, the activity loss was biphasic - an initial rapid loss during the first week, followed by a considerably slower decline after that. This may be due to residual water loss during the initial week at elevated temperatures.

1.3.5.2 Liquid state

Understanding MR vaccine activity in the liquid state may not apply to the dried MN patch, but maintaining activity as a liquid may enable a more efficient patch manufacturing process. Rather than using the traditional cellular $TCID_{50}$ assays, liquid formulations were screened by their prevention of aggregation measured by dynamic light scattering or by use of a measles virus which produces green fluorescent protein (MV-

GFP). In general, proteins (i.e. gelatin), sugars (i.e. trehalose, lactose, dextrose), polyalcohols (i.e. mannitol, sorbitol), and amino acids (i.e. lysine, valine) with neutral buffers were able to stabilize liquid measles vaccine at elevated temperatures [75, 76].

In order to fabricate patches, vaccines with high titers are necessary to load a full human dose in the needles. For this, preparations of highly purified, infectious viruses must be performed using gentle conditions. Tangential flow filtrations and diafiltration can be used to isolate vaccines; however ultracentrifugation has resulted in high loses of viral activity [71, 77]. The pH needs to be maintained between 5 and 9 to retain activity [77].

1.3.6 Maternal Antibodies

Maternal antibodies inhibit early MR vaccination and lead to a window of susceptibility. Infants are vulnerable to many infectious diseases while their immune system develops. To protect them early in life, maternal antibodies are passed to offspring [78]. These antibodies are present for approximately 6-9 months after birth. Between the waning antibody levels and vaccination, the infants are susceptible to disease; approximately one third of global measles cases occur before 9 months of age [79]. In developed countries with herd immunity, children receive their first dose of MMR at 12-15 months, which results in a 95% seroconversion rate [80]. Infants in developing countries vaccinated at 6-9 months have a much lower 60% seroconversion rate [80].Vaccination at 6 month produces low humoral immunity, but comparable t-cell interferon ɣ response [81, 82].

1.3.6.1 Infant Rhesus Macaque Model

Rhesus macaques have been used as a model for measles vaccination and infection because they share similar clinical reactions as humans [83]. In order to mimic maternal antibodies, an infusion of measles immunoglobulin can be infused prior to vaccination to induce similar humoral response as humans [84]. Newborn macaques receiving measles immunoglobulin had suppressed humoral and cellular immunity after vaccination [82]. DNA vaccination administered with IL2 adjuvant induced a potent, protective immune response [85, 86]. However, without the IL2, the DNA vaccine had a poor response compared to the live-attenuated vaccine in naïve infant rhesus macaques [87].

1.3.7 Microneedle Patch

Previous work in the Prausnitz lab and CDC has developed a measles vaccine microneedle patch. This patch was formulated with sucrose and threonine to maintain activity. The patch could be stored for four months with no loss at 5˚ or 22˚C and one log loss at 40˚C [88]. This patch induced potent immune responses in both cotton rats and rhesus macaques that were equivalent to subcutaneous injection [20, 88]. Finally, the patches were safe; no adverse events were noted in either animal model.

1.4 Extended Delivery

1.4.1 Benefits of Extended Presentation of Antigens

Vaccines which mimic natural infection kinetics, such as MR, can induce life-long protection from a single dose. However, newer vaccines, typically killed vaccines, often have better safety profiles but are often less effective [89-92]. These vaccines typically require multiple doses in order to achieve high seroconversion rates. Adjuvants such as

alum can improve the immune response [93]; however, few adjuvants are approved for use in vaccines [94]. Repeat vaccinations especially in developing countries are difficult to achieve and often leave coverage gaps. Because multiple doses must be spread months apart, 10 million children are under-vaccinated [39]. Thus, a single-dose vaccine could decrease the vaccination costs and increase vaccination coverage by eliminating booster shots [93].

The concept that a controlled release of a vaccine may induce a stronger immune response compared to a bolus injection has been around since 1979 [93, 95]. Most of the work with antigen controlled release systems centers on poly(lactide-co-glycolide) (PLGA) microparticles. PLGA particles have been used to deliver tetanus vaccine [96-98] and hepatitis B vaccine [99-102] and have, in general, been shown to be superior to bolus delivery of antigen or alum-adsorbed antigen [39]. The size of the microparticle and the site of administration influence the immune response [93]. Two issues have hindered the translation of this technology: (1) the instability of the encapsulated antigen [103, 104] and (2) the profitability of such vaccines [93]. Other methods for controlled release including gelatin microspheres [93], hydrogels [105], chitosan microparticles [106], and chitosan gels [107] have successfully improved the immune response. Additionally, implants and osmotic pumps provide more control over the release profile of the vaccine compared to injected particles [104, 108, 109]. Most of the systems described above utilize similar release profiles with an initial burst followed by low levels of release; these studies demonstrate that longer presentation can increase the immune response, yet they cannot determine the optimal kinetics or fully elucidate why controlled release works better [89]. Controlled release of cancer antigens resulted in the accumulation of CD8+ T cells at the

site of vaccination rather than at the tumor site, limiting the vaccine effectiveness; more short lived formulations induced a more favorable outcome [110]. All work presented here will center on vaccines for prophylactic prevention of infectious diseases.

1.4.2 Identification of Optimal Release Profile

While many studies have demonstrated the benefits of continuous release of an antigen, few have studied the antigen kinetics that induce the most robust immune response [39]. Some studies have attempted to identify the optimal release profile by utilizing different types of controlled release systems. Different conclusions were reached; however, because different systems of various sizes (i.e. PLGA microparticle vs liposomes) were used, it is hard to determine if changes in immune response were due to the material or the kinetics [111, 112]. Another approach is to use daily injections that mimic different release profiles [92]. Using this method, confounding factors such as material, antigen stability, and host-material interaction are removed.

Johansen et al. delivered a fixed dose of antigenic peptides and CpG as an adjuvant [92]. An exponentially increasing profile induced the highest CD8+ T cell responses compared to constant stimulation or bolus vaccinations. The number and activation levels of the dendritic cells were equivalent across groups, but the exponential immunization prolonged T cell proliferation and delayed dendritic cell recruitment [92]. Delivering human immunodeficiency virus antigens via subcutaneous injection or osmotic pump, Tam et al. demonstrated that exponentially increasing doses over two weeks induced highest antibody titers [109]. Using *in vivo* models and computation models, the authors concluded that the extended antigen presentation resulted in antigen retention in the draining lymph

nodes lead to an increase in B-cell differentiation in the lymph nodes and higher-affinity antibodies. In smaller studies, hollow MNs were used to deliver inactivated polio vaccine (IPV) into the skin with a constant or exponentially increasing profile for 4 or 8 days. Equivalent increases in IPV-specific IgG were seen across all extended delivery groups, but no differences between the different profiles were evident [113]. Finally, Jansen et al. was the only study that did not demonstrate an increase in immune response with daily injections compared to a bolus vaccination. This maybe be due to administering the vaccine in alternate sides of the chicken each day [114].

1.4.3 Slow Release from Microneedle Patches

MN patches have been used to deliver controlled release vaccinations into the skin. PLGA with vaccine cast into the MN tips or loaded as particles can be rapidly delivered into the skin [115, 116]. When such patches were tested *in vivo*, they improved the cellular response and induced comparable serum antibodies to bolus vaccination [115]. Chitosan MNs on a non-dissolving pedestal delivering ovalbumin over at least two weeks showed increased antibodies post vaccination [14]. MN patches containing poly(acrylic acid) and silk released ovalbumin for over two weeks. The release profile was an initial boost followed by low release over two weeks. *In vivo,* these MN patches increased proliferation of antigen-specific CD8+ T cells, prolonged local inflammation, and heightened the humoral response [117]. Furthermore, repeated application of dissolving MN patches in mice did not prompt any undesirable side-effects in the skin [118]. This may be a method for multiple deliveries of vaccines.

1.4.4 Vaccines Utilized

This work studies three other vaccines besides MR which was previously discussed: IPV, tetanus toxoid, and influenza. In all of these cases, the vaccine is an inactivated viral particle or protein; these vaccines do not induce potent, long lasting immune responses after a single dose. The eradication of polio is quickly approaching, with only 342 cases worldwide in 2014 [119]. To attain eradication, IPV must be incorporated in global vaccination campaigns. IPV is a formalin-inactivated vaccine that has three serotypes, each of which must be vaccinated against [120]. IPV requires at least three doses to meet high seroconversion levels [119]. Each dose costs at least about three dollars and is administered with a needle and syringe [120].

Clostridium tetani spores in the soil and environment can enter the body through cuts or abrasions on the skin where the bacteria can replicate and release toxoid, resulting in damaging neurological effects [121]. The WHO recommends six doses of tetanus toxoid vaccine spread from infancy to adolescence, followed by an additional boost every ten years [122].

Finally, subunit influenza vaccine is administered each year to prevent seasonal flu. In the 2015-2016 influenza season, the vaccination prevented approximately 5 million illnesses, 2.5 million medical visits. Due to antigenic changes, each year, three different strains are selected to be in the vaccine, and the population must be re-vaccinated [123]. The vaccine efficacy for influenza varies year-to-year but hovers around 50-60% [123]. For each of these vaccines, novel vaccine technologies that improve the immune response to a single dose could improve coverage and disease protection.

CHAPTER 2. OVERVIEW OF AIMS

2.1 Aim 1: Thermostability of Measles-Rubella Vaccine in a Microneedle Patch

In this aim, we hypothesize that the optimization of excipients using a screening process will result in a formulation that stabilizes live-attenuated measles and rubella vaccines during the microneedle patch manufacturing and storage. Vaccine stability when stored at elevated temperatures is crucial to the introduction of patches for developing countries. The removal of the cold chain will enable simpler house-to-house vaccination campaigns, reduce vaccine waste, and decrease the costs associated with vaccine administration. To achieve this goal, the effects of buffer and pH on vaccine activity during drying were studied. While measles vaccine is not susceptible to pH from 6.0 to 8.0 during drying, drying rubella vaccine at pH lower than 6.5 had a significant loss of activity; drying at pH 7.5 was optimal for maintaining stability. We then screened buffers with the capacity to buffer at pH 7.5; an overnight drying experiment showed potassium phosphate buffer to be suitable to both vaccines. Excipients such as sugars, amino acids, and proteins were chosen from the literature and previous lab experience. Two consecutive experiments tested the excipients stabilize potential during an overnight drying step and then during accelerated storage at 40˚C for one week. A smaller subgroup of excipients was tested in pairwise manner for stability for up to one week at 40˚C. Sucrose-threonine was identified as the top combination. Finally, vaccine microneedle patches fabricated with sucrose-threonine maintained vaccine activity for at least one month at elevated temperatures and were able to withstand freeze thaw cycles. The thermostable MN patches developed in this work will decrease vaccine waste, allowing for cheaper, easier vaccinations.

2.2 Aim 2: A Microneedle Patch for Measles and Rubella Vaccination is Immunogenic and Protective in Rhesus Macaques

The second aim centers on the immune response to microneedle patch delivery of live attenuated measles and rubella vaccine in rhesus macaques. The central hypothesis is that microneedle patches would induce a comparable immune response compared to subcutaneous injections which are the traditional route of vaccination. In the first study, juvenile rhesus macaques vaccinated with microneedle patches or subcutaneous injection showed comparable neutralizing antibody and IgG titers against both vaccines. The secondary purpose of this aim is to study the immune response in the presence of maternal antibodies. For this, infant macaques born to measles-naive mothers were vaccinated with or without an infusion of measles IgG. Animals vaccinated without maternal antibodies developed strong immune responses that later protected against a live measles viral challenge. Those vaccinated in the presence of maternal antibodies produced inferior responses and displayed signs of infection after viral challenge. Thus, microneedle patches induce a potent immune response that is non-inferior to subcutaneous vaccination.

2.3 Aim 3: Extended Delivery of Vaccines to the Skin Improves Immune Response

In the final aim, we examine the effect of vaccine extended delivery and presentation on the immune response. We hypothesize that vaccine release profiles that mimic natural infection kinetics will induce potent immune responses and that these profiles can be presented utilizing daily injections or microneedle patches. Through studies using inactivated polio vaccine and daily intradermal injections, we identified that a constant profile over one month induced the highest neutralizing antibody titers of the

profiles we tested. This principle applies to other inactivated or dead vaccines such as tetanus toxoid vaccine or subunit influenza vaccine; however, no improvement was seen when vaccination with live-attenuated measles vaccine. Finally, microneedle patches containing influenza vaccine were administered to mice over the course of a month; the animals displayed high hemagglutinin-inhibition titers compared to bolus vaccination or intradermal extended delivery injections. Extended delivery with microneedle patches elicited heightened humoral and cellular responses compared to bolus vaccination. Together, this aim develops a novel technique of utilizing extended delivery to improve immune response to vaccination.

CHAPTER 3. THERMOSTABILITY OF MEASLES-RUBELLA VACCINE IN A MICRONEEDLE PATCH

Measles and rubella vaccinations are highly effective at reducing disease prevalence; however, high costs related to administration and vaccine waste limit the extent of vaccine coverage. Microneedle (MN) patches can increase coverage by easing logistics and reducing costs. Here, we demonstrate the thermostability of a bivalent measles and rubella vaccine MN patch. We identified the need for a buffer during drying for rubella vaccine; potassium phosphate buffer at neutral pH was optimal for both vaccines. After screening 43 excipients for their ability to retain activity during drying and storage, a smaller subset of excipients was selected, and excipients were dried in pairwise fashion. Dried vaccine was stored for one week at 40°C; numerous combinations were able to maintain activity of both vaccines over this time. MN patches were fabricated with a sucrose-threonine-potassium phosphate buffer formulation and stored at various temperatures. These patches had no significant loss of activity after one month at 40˚C and had not passed the one log cut off after four months at lower temperatures. This work demonstrates the potential for MN patches to be removed from the cold chain, thereby decreasing vaccine cost and waste and increasing vaccination coverage.

3.1 Introduction

Elimination efforts rely on vaccines to protect the population, and vaccination coverage levels of over 95% are necessary in order to impede viral transmission [124]. However, vaccination rates for measles and rubella have stagnated over the past several

years [2, 50], contributing to 114,000 measles deaths in 2014 [49, 50] and over 100,000 children born with congenital rubella syndrome (CRS) [51]. In 2012, the Measles and Rubella Initiative, a group of UNICEF, World Health Organization (WHO), Centers for Disease Control and Prevention (CDC), the United Nations Foundation, and the American Red Cross set regional elimination goals for 2015 [49, 53]. These goals were not achieved in part due to limitations of current vaccination technologies to reach all populations needing vaccination [49].

The measles and rubella (MR) vaccine is stored in lyophilized ten-dose vials and administered using a needle-and-syringe. This method is expensive and resource heavy, which limits access to vaccination to populations in developing-country or hard-to-reach communities [125]. The lyophilized vials must be reconstituted properly before administration, requiring a trained health care professional. The needle and syringe used to administer the vaccine must be properly discarded after use to prevent injury to the patient or health care worker. In a recent vaccination campaign, 15 children died due to improper vaccine handling and needle re-use by untrained personnel [38]. The vials must be thrown out six hours after reconstitution, leading to notable levels of waste [32]. Finally, the need to maintain the cold chain is expensive and leads to vaccine wastage. The current lyophilized vaccine is stable at 4˚C for up to two years but only four weeks at 37˚C [126]. Breaks in the cold chain may lead to vaccine failures and potentially disease outbreaks [127, 128]. Due to gaps in the cold chain, approximately half of the vaccine vials had below the minimum potency dose when delivered to Brazil [129] or Nigeria [130]. One review estimated that 75-100% of shipments are exposed to freezing temperatures [131]. Once reconstituted, within one hour, approximately half of the activity is loss if the vaccine is

stored at 20˚C, while almost all of the activity is lost if kept at 37˚C [32, 126]. Therefore, vials must be discarded after 6 hours due to this loss of potency. The wastage factor or the number of vaccine doses wasted per dose administered is estimated at 3.4 for routine vaccination and 1.1 for supplemental immunization activities (SIAs) [132]. WHO requires novel MR vaccines technologies to withstand storage for one week at 37˚C without loss of more than one $\log_{10}$ of activity [49, 74, 133]. Due to the complexity of administration, house-to-house vaccination campaigns are limited, despite their ability to increase vaccination coverage [134]. Therefore, in order to reach the next set of regional and global elimination goals, there is a need for a novel vaccination technology that is single dose, eliminates needles, easily administered, and thermostable [32].

Microneedle (MN) patches provide a novel vaccine delivery method with the potential to increase vaccination coverage. MN patches consist of an array of water-soluble, solid, conically shaped needles attached to a patch backing [8, 10, 12, 115]. Each microneedle contains the vaccine entrapped in polymers, sugars, and other excipients [135]. Once these sharp needles painlessly pierce the skin, they fully dissolve, deliver the vaccine, and result in no sharps waste [4, 5, 26, 136, 137]. MN patches are available in single dose-single use packaging, reducing vaccine waste and easing administration [32]. Once dissolved, the microneedles cannot be re-used, which increases the safety of the vaccinator and the patient and reduces the waste that must be moved from the vaccination site. Additionally, these patches can be administered by minimally trained personnel, again reducing costs associated with each vaccination [20, 32, 33]. Finally, because the vaccine is stored in the dried state, vaccines generally show superior thermostability compared with liquid or lyophilized vaccines [9, 28, 29, 88, 138]. However, each vaccine that is

incorporated into a MN patch requires its own formulation to stabilize the vaccine during patch manufacturing and storage [29, 30].

The current MR vaccine is packaged in lyophilized vials. These vials must be shipped between 2 and 8˚C. Other methods of measles vaccination have employed carbon dioxide-assisted nebulization with bubble dryer (CAN-BD) or spray drying to stabilize measles vaccine. CAN-BD creates a dry powder with about half the water content of lyophilized vaccines that can be delivered via aerosol into the lungs. Measles vaccine powder with myo-inositol and sugars saw a 0.6 $\log_{10}$ loss during storage for one week at 37˚C [73, 139]. Spray drying can increase manufacturing throughput compared to lyophilized vials but must be reconstituted before administration. A combination of sucrose, trehalose, arginine, human serum albumin, and divalent cations stabilized measles vaccine for 8 weeks at 37˚C for one $\log_{10}$ loss with no loss observed over that time at 4˚C [74]. To our knowledge, no study has been performed on the stability of both vaccines together.

In previous work, MN patches were developed to deliver measles vaccine. These patches were thermostable with one $\log_{10}$ loss of activity after four months at 40˚C and were immunogenic in cotton rats and rhesus macaques [20, 88]. As global measles eradication efforts ramp up, there is a focus to include rubella vaccinations as part of the measles vaccination campaigns [125]. To that end, novel formulations should be developed to incorporate both measles and rubella vaccines into a single MN patch. Here, a methodical screen was used to identify a formulation that maintains stability of a bivalent MR patch during manufacturing and at elevated temperatures for at least one month. We

also demonstrated that these patches exceed the WHO requirements for novel MN vaccine technologies.

3.2 Materials and methods

3.2.1 Vaccines

MR vaccines were prepared as described previously [20]. Briefly, stocks of monovalent measles or rubella vaccines (generously provided by Serum Institute of India, Pune, India) were added to confluent flasks of Vero cells (ATCC Manassas, VA) with Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) and 2% fetal bovine serum (FBS, Gibco) and incubated for five days at 37°C. Cellular suspensions were freeze-thawed and centrifuged to remove the cellular debris. Measles vaccine titers were approximately 7 $\log_{10}$ $TCID_{50}$/ml, and rubella vaccine titers were approximately 5 $\log_{10}$ $TCID_{50}$/ml. Vaccine aliquots were stored at -80˚C until use.

3.2.2 Sample preparation and storage

Measles and rubella vaccines were formulated into casting solutions to be used in the MN patch fabrication. All excipients were purchased from Sigma Aldrich (St. Louis, MO). Measles vaccine was used as prepared at a titer of 7 $\log_{10}$ $TCID_{50}$/ml. Rubella vaccine was concentrated using Vivaspin filters (Sartorius, Goettingen, Germany) with a 300 kDa molecular weight cut off until the volume decreased ten-fold. Measles or rubella vaccine was mixed with excipient solutions to achieve final casting solution concentrations of 10% w/v excipient and 1% w/v carboxyl-methylcellulose (CMC) in buffer. If the solubility of

the excipient was lower than 10%, the maximum solubility was used instead. For pH and buffer screens, casting solutions containing 10% sucrose and 1% CMC were used.

As an initial screening process, 50 µl casting solutions of different formulations were cast into Eppendorf microcentrifuge tubes. These samples were dried overnight under vacuum with desiccant at room temperature (20-25°C). The following day, samples were packaged in sealed aluminum pouches (Oliver-Tolas Healthcare, Grand Rapids, MI) with desiccant and placed in stability chambers (Caron, Marietta, OH) at 5, 25, or 40°C.

3.2.3 Microneedle patch preparation

MN patches were prepared as previously described [9, 88]. First, MN patch molds were prepared, as previously described [140]. Then a casting solution was cast onto PDMS molds. The solution was dried into the tips of the cavities under vacuum, and excess solution was removed. A second cast of 28% w/v poly-vinyl alcohol (PVA, Acros Organics, Geel, Belgium) and 21% w/v sucrose was added to the molds. Following two days of drying at room temperature with desiccant, patches were demolded and packaged with desiccant as described above for storage in stability chambers at 5, 25, or 40°C.

3.2.4 Measles infectivity assay

Samples were reconstituted in 1 mL DMEM. Tenfold dilutions of the sample were added to Vero cells seeded on 48 well plates with DMEM with 2% FBS. After five to seven days of incubation, plates were incubated with crystal violet, and titers were counted using the Spearman and Karber algorithm [141].

3.2.5 Rubella infectivity assay

To determine the rubella vaccine titer, samples were reconstituted in 1mL DMEM [70]. If the sample contained measles vaccine, anti-measles IgG (EMD, Millipore, Billerica, MA) was incubated at a 1:500 dilution with the sample for 1 hour at 37˚ C. Then, tenfold dilutions of the sample were incubated on Vero cells and incubated for one hour at 37˚ C followed by the addition of a mixture of DMEM, avicel (FMC BioPolymer, Newark, DE), and FBS. After three to five days, cells were fixed in cold methanol, incubated with E1 antibody (CDC, in-house), HRP-conjugated antibody, and precipitating TMB (Clinical Science Products, Mansfield, MA). An ELISPOT analyzer (CTL, Cleveland, OH) was used to count the foci.

3.3 Results

3.3.1 pH and buffers

We first wanted to determine the effect of pH on measles and rubella vaccine stability during drying. Potassium phosphate buffer was selected due to its ability to buffer across the selected pH range of 6.0 – 8.0. After drying, there was no significant loss of measles vaccine activity after drying over the pH range studied (Figure 3.1, top, ANOVA, $p=0.764$). On the other hand, rubella vaccine activity was significantly lower after drying at low pH or in unbuffered water (ANOVA, $p<0.0001$). Drying at pH 7.0 to 7.5 was significantly improved compared to drying in water but not statistically different from one another ($p=0.999$ and 0.447 for measles and rubella, respectively). Drying at either pH resulted in no loss of activity for rubella vaccine but some loss for measles vaccine (dotted line).

Although potassium phosphate buffer was effective, we next wanted to optimize the choice of buffers. Six buffers with buffering capacity around pH 7.0 and 7.5 were selected and screened for activity after drying. For measles vaccine, M199 and histidine buffers had lower activity compared to the other four buffers (Figure 3.1, bottom, ANOVA, p=0.031). For rubella vaccine, M199 and ammonium acetate buffers had lower activity upon drying; an ANOVA analysis comparing DMEM, HEPES, and potassium phosphate showed that they were not significantly different in their ability to stability measles or rubella vaccines (p=0.408). Based on these data combined, potassium phosphate was selected as the buffer to use in subsequent experiments because it showed good results with both vaccines and is the buffer currently used in commercial MMR vaccines [142].

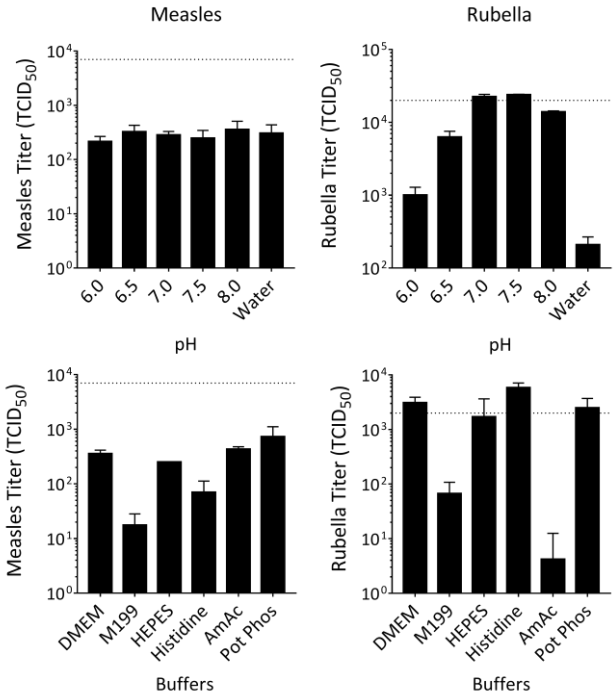

Figure 3.1. pH and buffer.
Samples show increased vaccine activity when dried at neutral pH. (TOP) Monovalent vaccine samples were dried in potassium phosphate buffer over a pH range or deionized water. Rubella vaccine loses activity when dried at low pHs.

(BOTTOM) When dried in a variety of buffers, DMEM, HEPES, and potassium phosphate all performed equivalently. Dotted lines indicate liquid controls (n=2 or 4).

3.3.2 Single excipients during drying

Forty-three excipients, such as sugars, amino acids, proteins, and salts, were selected from literature and ingredients lists of currently approved vaccines. Each excipient in potassium phosphate buffer was mixed with the vaccine, and solutions were dried overnight in Eppendorf microcentrifuge tubes under room temperature vacuum and in the presence of desiccant. The remaining activity of rubella vaccine after drying (Figure 3.2, grey bars) shows that many excipients were able to maintain measurable level of activity. However, measles vaccine was more susceptible to damage due to drying, and many fewer excipients were able to maintain measles vaccine activity after drying (black bars). Excipients that maintained activity above the detection limit for both vaccines were selected to move onto the next phase.

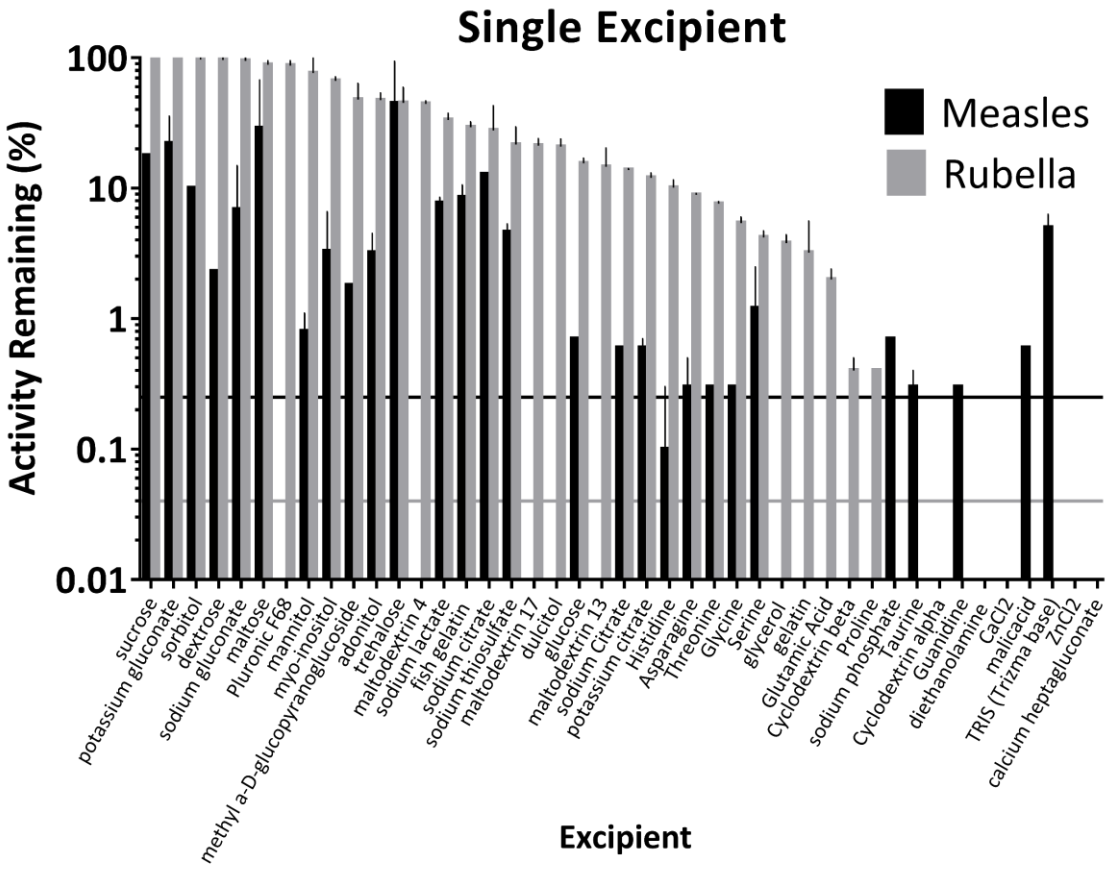

Figure 3.2. Screen excipients in overnight dry.
Excipients were mixed with monovalent vaccine, dried overnight at room temperature, and reconstituted for analysis. The solid lines represent the lower detection limit for each assay (black for measles, grey for rubella). Excipients that did not retain any activity were eliminated for the next phase (n=2).

3.3.3 Single excipients during storage

We next studied the effect of excipients on thermostability of measles and rubella vaccines during storage at elevated temperatures. Single excipients were mixed with vaccine, dried overnight at room temperature, and then stored at 40˚C with desiccant. After seven days, only three excipients had detectable measles vaccine activity: histidine, sucrose, and trehalose. For rubella vaccine, roughly two thirds of the excipients had some activity by day 7. There are two sources of activity loss: drying and storage. Certain

excipients were effective at maintaining stability during drying. To separate the two effects, activity at day 0 was compared to the liquid control representing no loss of activity (dotted line, Figure 3.3 and Table 1). From this analysis, several excipients such as sorbitol, dextrose, and gluconate, were selected for the next round. Other excipients were more ideal for reducing activity loss during the storage at elevated temperatures. The viral activity after one week was compared to the activity after drying at day 0. From these percentages, the top excipients for storage were selected: histidine, sucrose, trehalose, and asparagine.

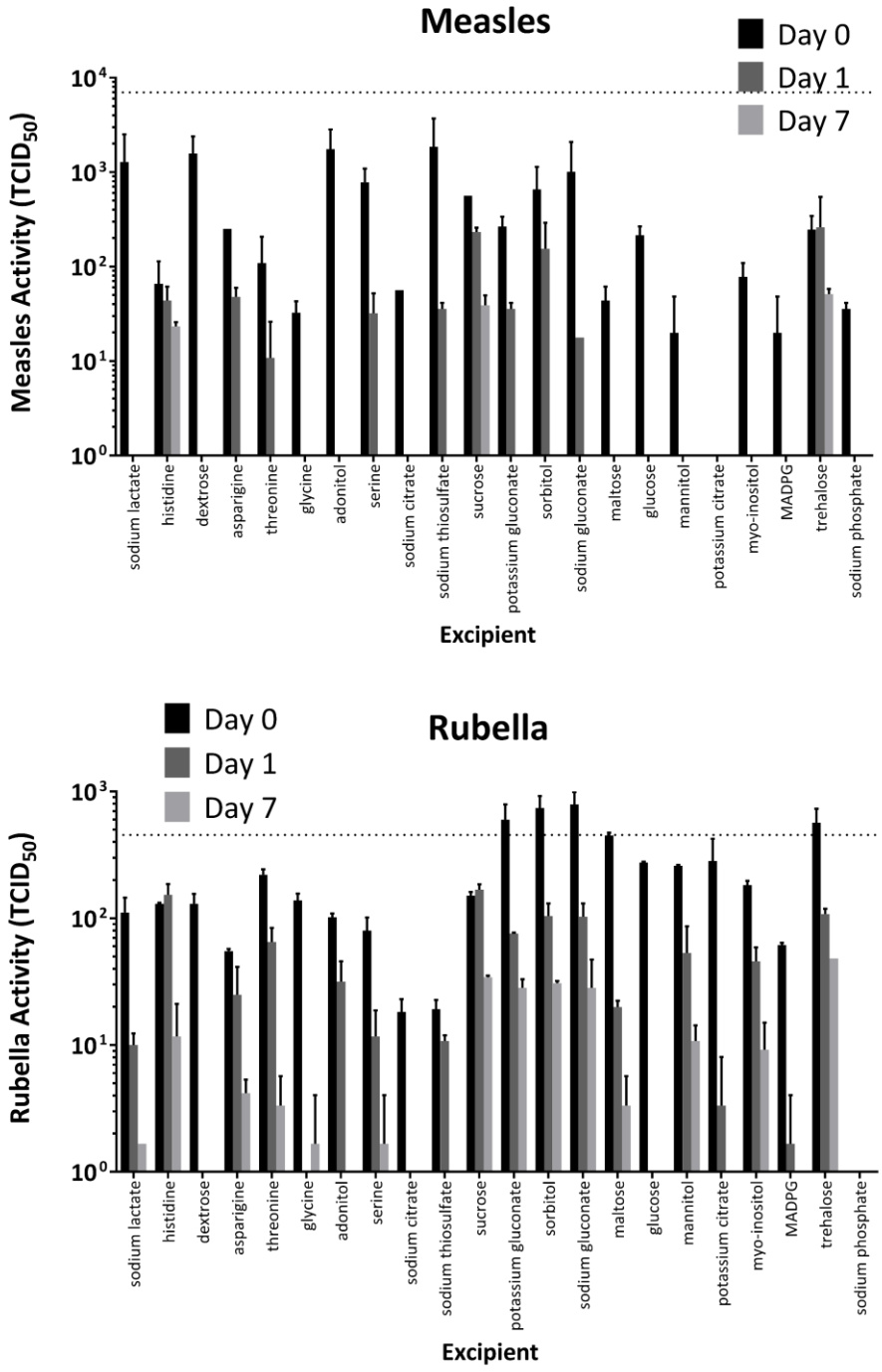

Figure 3.3. Single excipients storage screen.
After drying, samples were stored at 40°C for up to one week. The dotted lines represent 100%. During storage, only three excipients (histidine, sucrose, and trehalose) were able to maintain any measurable level of activity (TOP). For rubella, most of the excipients were able to maintain some activity (n=2).

3.3.4 Combination of excipients

We hypothesized that by combining excipients, certain pairs of excipients would demonstrate better stability than either of the individual excipients alone. Stabilizers from the previous screen were selected for their ability to stabilize during drying and/or during storage. The stabilizers were combined in pairwise fashion and compared the titers at day 0 and day 7 after storage at 40°C to two controls: sucrose-threonine, which was the excipient formulation used in a previous study of measles vaccine MN patch stabilization [88] and the liquid control (dashed line, Figure 3.4, Table 2, and Table 3). Three excipient combinations, histidine - sucrose, histidine - sodium gluconate, trehalose - asparagine, showed no statistical loss in activity between stock - day 0 and day 0 - day 7 (two t-tests, $p>0.05$). We also compared the remaining activity at day 7 to the activity of sucrose - threonine at day 7. Several combinations had higher activity for either measles or rubella vaccine, but no combinations had higher mean titers for both vaccines. In general, sugars, such as sucrose and trehalose, were good stabilizers for drying and storage of both vaccines. Histidine was a particularly good stabilizer during drying for measles vaccine. Taken together, using a screening process, we identified excipient formulations that can retain activity of both measles and rubella vaccines for at least one week at 40˚C.

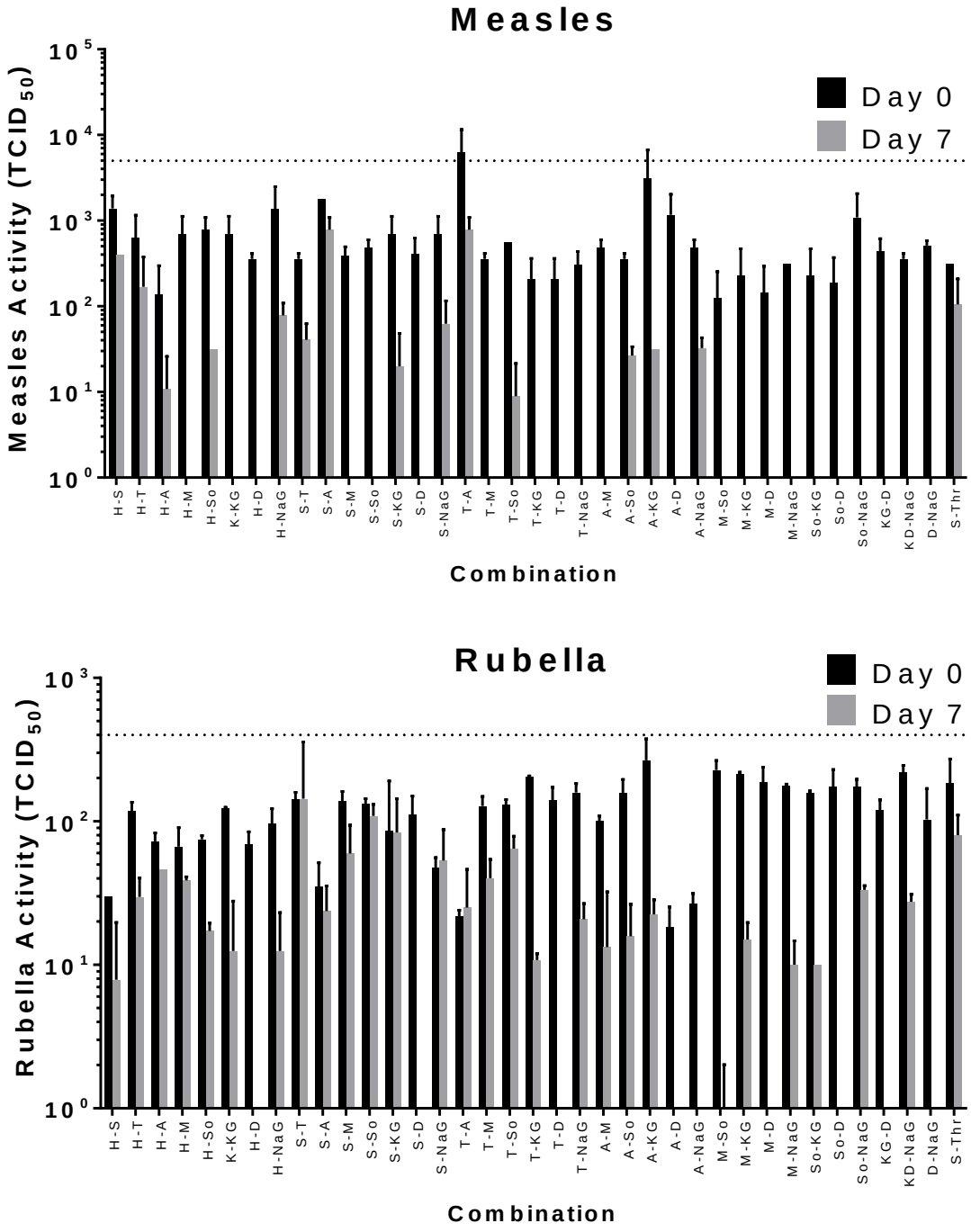

Figure 3.4. Combinations of two excipients screen.
Pairwise combinations of excipients were mixed with monovalent vaccine, dried overnight, and stored with desiccant at 40°C for one week. Titers after drying (Day 0, black bars) and after storage (Day 7, grey bars) are represented for each vaccine. The dotted lines represent 100% activity for each vaccine. Combinations outperform their single excipient components (n=2).

3.3.5 Storage in microneedle patches

Until now, stability was studied by casting solutions into tubes. In the final study, we wanted to look at the stability of MR vaccines in MN patches over a range of temperatures. MN patches were fabricated with 10% sucrose, 3% threonine, and 1% CMC in potassium phosphate buffer. Patches were stored at 5, 25, or 40˚ C with desiccant. After one month, no statistical difference was noted at any temperature for any vaccine (Figure 3.5, t-test, $p>0.01$). By the two-month time point, the rubella vaccine titer was statistically lower at all time points than day 0 ($p=0.0078$, 0.0029, and <0.0001 for 5°, 25°, and 40°C respectively), while measles vaccine remained stable for 4 months. For the first time, we have shown that a MR vaccine microneedle patch is stable at elevated temperatures for at least one month. This surpasses the WHO requirements for stability at 37˚ C for one week.

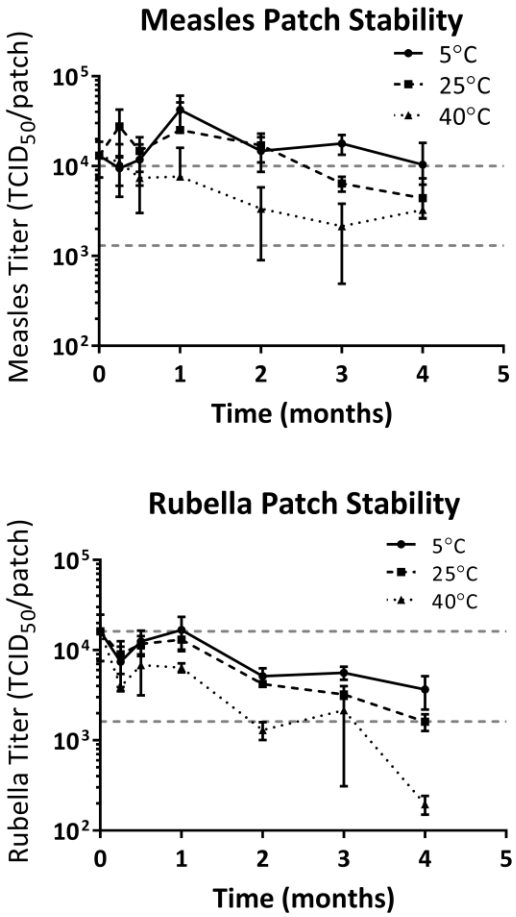

Figure 3.5. Microneedle patches were stable at elevated temperatures.
Bivalent microneedle patches were fabricated with sucrose and threonine and stored at 3 temperatures. No loss of activity was seen over the first month of storage (n=4).

3.3.6 Freeze thaw cycles

During vaccine transportation in the cold chain, vaccines may experience fluctuating temperatures. If packaged adjacent to ice packs, vaccines may be exposed to near freezing conditions. To test vaccine stability in these cases, MN patches were exposed to freeze thaw cycles, where temperatures shifted from 5°C to -20°C every hour and a half. After one or three cycles, patches were stored overnight at 5°C. The resulting titers were normalized by the titer for patches stored at 5°C (Figure 3.6). While there was some loss

in the measles vaccine titer, no statistically significant differences were detected between the groups (ANOVA, p=0.25).

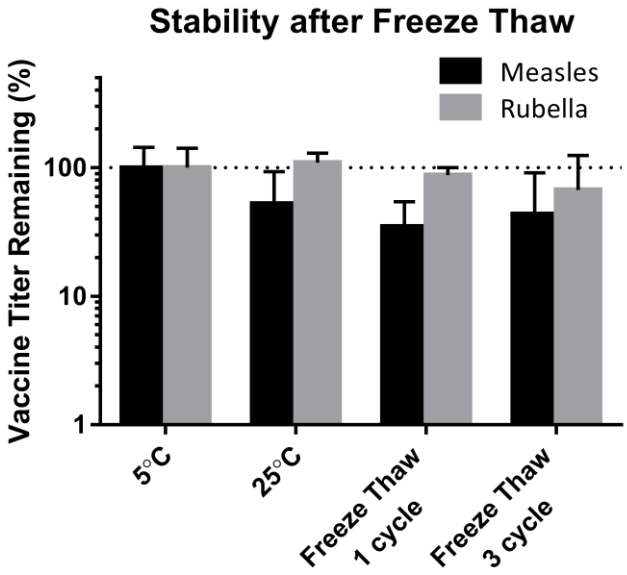

Figure 3.6. Stability following freeze thaw cycles.
MR MN patches were fabricated and packaged with desiccant. Vaccine titers were analysed after storage at 5°C, 25°C, one freeze thaw cycle, or three freeze thaw cycles. Titers were normalized by the activity of patches stored at 5°C. Although there was loss detected in the measles titers, here was no statistical difference across any of the groups (ANOVA, p=0.25, n=4).

3.4 Discussion

The licensed measles and rubella (MR) vaccine is safe and highly effective at prevention of disease. However, the current technology to deliver the vaccines faces numerous challenges, limiting the widespread use of the vaccine and hindering elimination efforts. To our knowledge, this is the first time that the thermostability of both measles and rubella vaccines has been studied simultaneously for the development of MN patches. We demonstrate that through a screening process we identified a formulation that kept both vaccines stable for at least one month at elevated temperatures. This surpasses the WHO

requirement for novel MMR vaccine products to lose up to one $\log_{10}$ of activity after one week at 37˚ C.

When we screened drying of the vaccines in buffers, the presence of a buffer is extremely important to maintain rubella vaccine activity during drying, unlike measles vaccine. The E1 and E2 proteins on the rubella virus membrane undergo a conformation shift between pH 5 and 6 [143, 144]. Once exposed to a low pH environment, the rubella virus shows a remarked reduction in infectivity [143]. Additionally, the pH of a solution drops as water is removed through crystallization during freezing or evaporation during drying [145]. The decrease in pH has been shown to influence protein conformations and result in denaturation [146, 147]. These literature observations are consistent with our finding that buffer at neutral pH is essential to maintain activity of rubella vaccine during drying.

In the next part of the screen, we studied the effects of various excipients to stabilize MR during drying and storage. During drying and storage, one of the key stresses on the proteins is the removal of the protein's hydration shell [148]. Sugars, such as sucrose, trehalose, and gluconate, could provide hydrogen bonding to the protein and serve as water substitutes during drying [148, 149]. Depending on their side chains, amino acids stabilize proteins in the dry state through a variety of mechanisms, including ionic interactions, reducing exposed hydrophobic regions, filling of void volumes, and slowing the molecular dynamics [150, 151]. We further demonstrated that solutions with two excipients outperformed the single excipients during drying and storage. This is consistent with other literature which show co-solutes are beneficial in protein stability [9, 88, 151, 152].

Microneedle patches offer many advantages over current needle and syringe delivery technology. MR elimination and possible future eradication efforts rely on high vaccination coverage to meet their goals; however, these efforts are hindered by the reliance of current lyophilized vaccines. MN patches remove many barriers in vaccination campaigns such as biohazardous sharps waste, single-dose packaging, and administration by minimally trained personnel.

Here, we demonstrate the development of a bivalent measles-rubella MN patch which can be stored at 40˚ C for up to one month with no loss of vaccine activity, with up to four months at 5°C with less than one $\log_{10}$ activity loss. These findings exceed WHO requirements for one $\log_{10}$ loss after one week at 37°C. While our findings may not allow for complete removal from the cold chain, it does suggest that patches could be transported in a controlled temperature chain, in which the 'last mile' may be conducted outside of the cold chain. During a vaccination campaign, patches could be refrigerated from manufacturer to central distribution points and district level sites. During the last phase in which vaccinators go house-to-house or which take place in remote areas, patches could be stored at ambient temperature without the need for any refrigeration. This contrasts with lyophilized vaccines. These vaccines must constantly be kept on ice even when accessing remote locations, as the ice is necessary to keep the reconstituted vaccine cold during the vaccinations. Thermostable MR MN patches may allow for a different vaccine vial monitor (VVM) to be used. Current lyophilized measles or MR vaccines are manufactured with a VVM14 for medium stability. This medium monitor allows for 14 days at 37°C, 90 days at 25°C, or 3 years at 5°C. If VVM30 were used, vaccine could be stored for longer times at mid to high temperatures, thereby reducing vaccine waste.

Increasing global vaccination coverage rates have drastically diminished the mortality and disease burden of MR. However, coverage rates have stagnated due, in part, to use of a needle and syringe with a lyophilized vaccine. This vaccine must be refrigerated during shipment, once reconstituted, can only be used for 6 hours. Here, we have demonstrated that measles-rubella vaccine microneedle patches can be stored at elevated temperatures without loss of activity. These patches may allow for partial removal from the cold chain. The added thermostability can decrease costs and logistics necessary for mass vaccination campaigns. Overall, if implemented in routine and supplemental immunization activities, MN patches can increase vaccine coverage and aid in elimination and eradication efforts.

3.5 Conclusion

In order to eliminate and eventually eradicate MR, higher vaccination coverage is necessary. The global health community has called for improved vaccine delivery devices that can ease administration, are thermostable, and do not require needles. To this end, we developed thermostable MR MN patches that can painlessly delivery vaccine into the skin. We studied the effects of pH and buffers on vaccine stability during drying, finding that potassium phosphate buffer at pH 7.5 to be optimal. Next, 43 excipients were narrowed down by their ability to maintain activity during drying and storage, and top excipients were tested as pairs. Sucrose-threonine- potassium phosphate MN patches were then tested for long term stability. After one month at 40°C, no statistically significant activity was noted, exceeding the WHO requirement for one $\log_{10}$ loss after one week. After two months, titers for rubella vaccine had fallen below that threshold. MN patches were not

susceptible to loss during freeze thaw cycles. In conclusion, the thermostable MR MN patches developed here can be used to increase vaccine coverage.

CHAPTER 4. A MICRONEEDLE PATCH FOR MEASLES AND RUBELLA VACCINATION IS IMMUNOGENIC AND PROTECTIVE IN RHESUS MACAQUES

Despite the availability of safe, efficacious vaccines, measles and rubella (MR) remain major public health concerns especially in developing nations with low vaccination coverage rates. New vaccination delivery methods may help increase coverage to allow countries to achieve regional elimination goals and enhance control of MR and congenital rubella syndrome. Microneedle (MN) patches consist of micron-scale projections composed of water-soluble materials, encasing the vaccines. These patches can be administered by minimally trained personnel, leave no biohazardous sharps waste, and remove the need for vaccine reconstitution. The goal of this study was to evaluate the immunogenicity of MR vaccination using MN patches in juvenile rhesus macaques and in infant rhesus macaques in the presence or absence of measles maternal antibody (MiG). In juvenile rhesus macaques, MN patches induced comparable immune response to subcutaneous injection (SC). In the infants in the absence of MiG, protective titers of measles neutralizing antibodies (>120 mIU/ml) were detected in 100% of macaques in the MN group and in 50% of macaques in the SC group post-vaccination. However, protective titers to measles were not detected in either of the groups that received MiG prior to vaccination. Rubella neutralizing antibody titers were in excess of 10 IU/ml for all groups. All macaques in the MN group were protected from measles challenge, while 75% were protected in the SC group; none of the macaques that received MiG were protected. These results show, for the first time, that a single dose of MR vaccine delivered by MN patch

generated protective titers of neutralizing antibodies to both MR in rhesus macaques and afforded complete protection from wild-type measles virus challenge.

4.1 Introduction

Measles and rubella have been effectively controlled in many parts of the world by achieving high levels of coverage with measles and rubella vaccine. However, global vaccination coverage has plateaued at approximately 85%, and measles and rubella continue to circulate in countries with inadequate coverage. Measles caused 135,000 deaths in 2015, and 100,000 children are born with congenital rubella syndrome each year. The World Health Organization (WHO) established regional vaccination coverage goals of ≥90% nationally and ≥80% in every district [47]. However, utilizing current vaccination methods, vaccination coverage has stagnated around 85% for the first dose of measles-containing vaccine and 56% for the second [49]. While this increased vaccination rates account for a 79% decrease in measles-attributed deaths over the past fifteen years, this decrease was insufficient to reach 2015 global goals [47]. Rubella had much lower global vaccination coverage of 46% in 2014 [49]. Congenital rubella syndrome (CRS) or the infection of a pregnant women and her child can result in miscarriage, fetal death, or disabling conditions such as heart disease, blindness, and deafness; increases in vaccination coverage have not alleviated the incidence of CRS over the past twenty years [51].

Smallpox eradication in 1980 and on-going polio eradication efforts over the past twenty-five years have been successful largely due to administration of the vaccine in house-to-house campaigns by millions of minimally trained volunteers and health care workers [153-155]. House-to-house campaigns can increase vaccination coverage 6-12%

compared to fixed vaccination sites. The Measles and Rubella Global Strategic Plan 2012-2020 Midterm Review states that these campaigns have the potential to increase coverage levels above herd immunity (>95%), thus enabling elimination goals to be achieved [49].

Current measles and rubella (MR) vaccination strategies, utilizing needle-and-syringe delivery with lyophilized vaccine in multi-dose vials, face numerous logistical challenges that limit the execution of house-to-house campaigns needed to reach programmatic vaccine coverage goals. First, the vaccine vials, reconstitution media vials, syringes, and needles must travel to remote vaccination sites. The vaccine must be kept between 2 and 8˚C throughout this journey to prevent viral activity loss [55]. Multi-dose vials cost less per dose and require less space in the cold chain, but account for significantly more vaccine wastage due to mishandling or excess [156]. By some estimates, approximately half of vaccine delivered to developing countries must be discarded due to cold chain breaks and wastage from multi-dose vials [138, 157]. During vaccination, trained health care workers (HCW) are required to administer the vaccine, placing a significant strain on the human resources necessary for vaccinations [49]. The reconstitution step adds potential error to ensure the proper volume is added without heat shock due to warm reconstitution media [31, 138]. Recently, fifteen children died in South Sudan due to measles vaccine reconstitution errors [38]. Finally, after vaccination, HCWs must safely dispose of used needles and syringes. Approximately three million HCWs are injured due to contaminated sharps each year, leading to 15,000 new cases of hepatitis C; 70,000 cases of hepatitis B; and 500 cases of human immunodeficiency virus [35-37]. These factors indicate the need to improve vaccine delivery technology in order to increase MR vaccination coverage.

Here, we introduce microneedle (MN) patches as a novel method to administer MR vaccine [5, 26, 158-160]. MN patches consist of an array of solid MNs, each several hundred microns tall. These MNs are made of vaccine encapsulated in water-soluble polymers, sugars and other excipients in a formulation that is strong enough to pierce the skin, where the MNs dissolve to release their vaccine cargo [10, 11, 115]. MN patches offer numerous benefits to vaccination campaigns. In particular, the patches are small in size (e.g. 1 cm^2) and do not require reconstitution media. Because the vaccine is in a dry state, encased in stabilizing excipients, patches can be stored at ambient or elevated temperatures for long periods of time without loss of vaccine potency [9, 28, 88, 161]. Patches can be administered by minimally trained personnel or even self-administered and are strongly preferred by patients over hypodermic injection [16, 33, 162]. Finally, because MNs dissolve in the skin, used patches cannot be reused and pose no risk of needle-stick injury to the patient or healthcare worker [26].

Maternal antibodies present another challenge to measles elimination. Infants of vaccinated or naturally-exposed mothers have high levels of circulating anti-measles antibodies that wane over the first six to nine months of life [80, 81, 163]. These maternal antibodies inhibit development of long-lasting immune response following vaccination [78, 164], and the time between low antibody titers and vaccination leaves infants susceptible to measles infection. Due to herd immunity present in developed countries, vaccination of infants can be delayed until 12-15 months of age, leading to a 95% seroconversion rate. In developing countries where infants are vaccinated at 6-9 months, the seroconversion rate is much lower at approximately 60% [80]. Therefore, elimination campaigns would greatly benefit from a novel vaccine technology that would allow

administration within the first six months of life, ideally at birth. MN patches deliver vaccines to the skin, an organ rich in resident antigen-presenting cells, such as Langerhans cells [5, 26, 158-160]. Intradermal vaccinations have been shown to exhibit dose sparing [41, 43, 44] and strong immune responses in vulnerable populations [42, 45, 165]. We hypothesize that skin vaccination with a MN patch can induce a potent immune response, potentially overcoming maternal antibodies.

In our previous work, MN patches loaded with measles vaccine were shown to be immunogenic in cotton rats and juvenile rhesus macaques, and exhibited no loss of potency when stored at 25˚ C with desiccant for up to four months [20, 88]. A key highlight of the Midterm Review is the incorporation of rubella vaccines into routine measles vaccinations. MR vaccine patches have not yet been fabricated or tested for immune response or thermostability. Furthermore, the immune response to any vaccine administered by MN patches has not been studied before in infant rhesus macaques. Infants have less developed immune systems and have been shown to develop lower antibody titers and avidity, compared to their juvenile counterparts [163, 166]. Finally, MN patches have not been in the presence of maternal antibodies, naturally occurring or mimicked using an infusion of measles immunoglobin [84]. This study sought to address these issues by studying the immune response to MR vaccination using a MN patch in juvenile and infant rhesus macaques.

4.2 Materials and methods

4.2.1 Vaccines

The Edmonston-Zagreb vaccine strain for measles and the RA-27 rubella vaccine were obtained from stocks at the CDC. Vaccines were passaged in Vero cells (ATCC, CCL-81, Manassas, VA) maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) and 2% fetal bovine serum (FBS, Gibco). Infected cells were harvested, freeze-thawed, and subject to low-speed centrifugation to separate the virus from cellular debris. Vaccine aliquots were stored at -80°C [20].

4.2.2 Production of MN patches

MN patches were prepared as previously described [9, 88, 167]. Briefly, measles vaccine, rubella vaccine and an excipient solution of sucrose, threonine, and carboxymethylcellulose (CMC, Sigma-Aldrich, St. Louis, MO) in potassium phosphate buffer (pH 7.5) were mixed and placed on a polydimethyl siloxane micromold with vacuum. The vaccine solution was allowed to dry into the tips of the MN cavities, and residual material on the micromold surface was removed via tape-stripping. A solution of polyvinyl alcohol (Acros Organics, Geel, Belgium), sucrose, and DI water was added. After two days in a desiccator at room temperature (20 – 25°C), MN patches were demolded and stored desiccated at room temperature until use.

To measure the efficiency of delivery, MN patches were inserted into pig skin *ex vivo* for 20 min to allow complete dissolution of the MNs. The skin was stained with gentian violet (Humco, Texarkana, TX) to visualize puncture holes. Viral titers were measured in fresh and used patches the difference in titers before and after insertion was used to estimate the delivered dose.

4.2.3 Stability of MN patches

MN patches were sealed in aluminum foil pouches (Oliver-Tolas Healthcare, Grand Rapids, MI) with desiccant. Pouches were stored in Model 6020 environmental test chamber at 40°C (Caron, Marietta, OH) for one month. At various time points, patches were removed from the chamber, and viral tires were measured.

To determine measles vaccine titer, tenfold dilutions of vaccine were inoculated onto confluent monolayers of Vero cells in DMEM with 2% FBS. Five to seven days later, cytopathic effect was detected after incubation with crystal violet. Titers were determined using the Spearman & Karber algorithm as previously described [141]. To determine rubella vaccine titer, vaccine was incubated for one hour with anti-measles IgG (EMD Millipore, Billerica, Massachusetts) at 37°C to inhibit measles vaccine infection. Tenfold dilutions of the vaccine-measles IgG solution were incubated on confluent layers of Vero cells for one hour at 37°C. A mixture of DMEM, avicel (FMC BioPolymer, Newark, DE) and FBS were added to each well. After three to five days of incubation, cells were fixed with methanol, incubated with E1 antibody, HRP-tagged antibody, and tetramethylbenzidine sequentially (NeA-Blue TMB substrate, Clinical Science Products, Mansfield, MA). Foci were counted using an ELISPOT analyzer (CTL, Cleveland, OH).

4.2.4 Juvenile rhesus macaques

Colony-bred macaques were housed at the CDC Animal Facility. At approximately two years old, the hair on a region on the back of each macaque was removed with clippers and Nair (CVS Pharmacy, Woonsocket, RI). Patches were inserted with the thumb without any external device. The patch was pressed into place for 30 sec and allowed to dissolve

for 15 min. For the SC group, the vaccination occurred in approximately the same region of the back. Serum was collected weekly for eight weeks.

4.2.5 Infant rhesus macaques

Colony-bred infant macaques used in the present study were housed at the California National Primate Research Center in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care International Standards. The Institutional Animal Use and Care Committee of the University of California, Davis, the Georgia Institute of Technology, and the Centers for Disease Control and Prevention approved these experiments. Infant macaques were co-housed with measles and rubella sero-negative dames and weaned 42 days (± 5 days) prior to vaccination. Animal vital signs were regularly monitored. For blood collection and virus inoculation, animals were anesthetized with 10 mg/kg ketamine hydrochloride (Parke-Davis, Detroit, MI) injected intramuscularly.

4.2.6 Infant rhesus macaque immunization, inoculation, and sample collection

Sixteen infant macaques (18-49 days old; mean body weight 0.5 kg) were assigned to one of four experimental groups (Table 4). As previously described [86], 4 ml measles immunoglobulin (MiG) were administered to two groups at a mean of 32 days (± 9.6 days) after birth and 48 hrs prior to vaccination. MiG-treated and untreated groups were administered MR vaccination by either subcutaneous injection (SC) or MN patch. For MN vaccination, patches were pressed onto the skin, and gentle pressure was applied for 30 sec. Patches were then left on the skin for 15 min to allow for MN dissolution. Serum

samples were collected every two weeks post vaccination (PV), except at 8, 16, and 26 weeks PV, when heparinized blood samples were collected.

At week 28 PV (week 0 post challenge, PC), all vaccinated infant macaques in addition to an unimmunized group of macaques (means age 365 days) were challenged by intranasal inoculation with measles virus MV12 $1x10^6$ $TCID_{50}$/ml grown in a Vero cell line. The derivation of the viral stock for the challenge is described in the Supplemental Methods. In methods previously described [86], nasopharyngeal washes and heparinized blood samples were collected on 0, 1, 2, 3, 4, 5, 8, and 12 weeks PC. Peripheral blood mononuclear cells (PBMCs) were obtained from all macaques on the day of challenge (day 0), and 7, 14, and 21 days after challenge.

4.2.7 Serologic methods

Serum antibody titers were determined using commercial ELISA kits: Measles IgG (Serion ELISA classic- Measles IgG, virion\serion, Würzburg, Germany), Measles IgM (Serion ELISA classic- Measles IgM, virion\serion), and Rubella IgG (Zeus ELISA IgG Test Kits, Branchurg, NJ).

Neutralization antibody titers to measles were determined using the standard plaque reduction neutralization (PRN) assay [168], and antibody titers were determined based on Third WHO International Standard Reference Serum (97/648). Neutralizing antibodies to rubella were measured using a focus-reduction assay as described previously [70]. A value of 1 IU/ml was assigned to all serum samples with titer less than 5 IU/ml.

4.2.8 PBMC Analysis

Measles viremia was measured by co-culturing dilutions of PBMCs with Raji cells as previously described [169]. In addition, the copy number of measles RNA in cryopreserved PBMCs was measured by modification of a previously described RT-qPCR assay [170]. Briefly, 10^5 PBMCs were thawed and centrifuged at 1500x*g* for 5 min, and RNA was extracted by using a RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA was prepared using random hexamer primers (ThermoFisher, Waltham, MA) and SuperScript III reverse transcriptase (ThermoFisher) per manufacturer protocol and diluted to 40 µl. The measles RNA gene was measured by RT-qPCR as previously described [170]. A constitutively expressed reference gene, GAPDH, was quantified to control for cellular/RNA input and quality of preparation. Copy numbers of the RNA coding for the measles nucleocapsid (N) gene were determined by interpolation of the average measured threshold cycle number onto a standard curve produced with a purified PCR amplicon containing a fragment of the N gene.

4.3 Results

4.3.1 Fabrication of microneedle patches

The MN patches used in this study consisted of an array of 100 MNs in a 10x10 grid of approximately 1 cm^2 mounted on a backing structure to facilitate handling (Figure 4.1a). The MNs were solid, conical structures made of water-soluble excipients and contained at least 1000 $TCID_{50}$ of measles and rubella vaccine (Figure 4.1b). The MN patches could be pressed onto the skin and, upon penetration into skin, the MNs dissolved, leaving behind only the base structure on which they were mounted (Figure 4.1c). The MN patches were thermostable (Figure 4.1d); when stored for up to one month at 40°C, there

was no significant loss of vaccine potency, which exceeds the WHO requirement for stability at 37°C for one week [51, 52]. For future use in vaccination campaigns, a packaging concept was developed in which MN patches were housed on blister trays housed in cardboard boxes (Figure 4.1e). In this configuration, each single-dose MN patch required 10 cm^3 of packaged volume and 4 g of packaged weight.

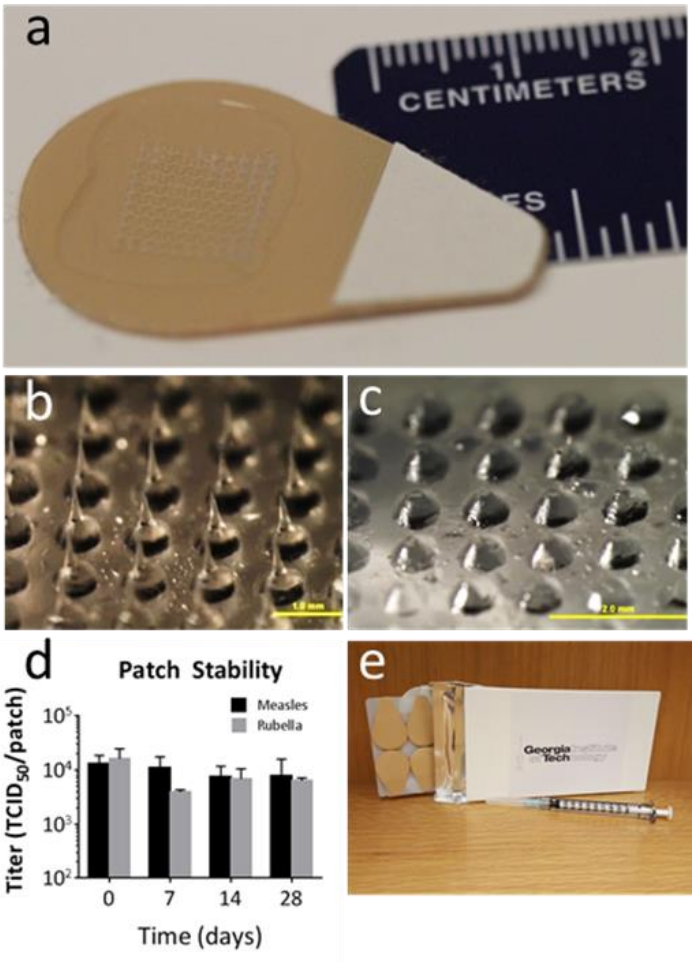

Figure 4.1. Measles-rubella vaccine microneedle patch.
Microneedle (MN) patches consist of micron-scale projections encasing measles and rubella (MR) vaccine in water-soluble excipients. (a) The MN array is 1 cm^2, and the entire patch is 3.5 cm by 2 cm in size. (b) The MNs are 700 µm long, and (c) once inserted, the needles dissolve completely, leaving only a blunt base structure and generating no sharps waste. (d) MN patches were stored at 40˚C for up to one month without significant loss in vaccine activity. Data show mean titer ± SD (n= 4 replicates). (e) A box containing 50 doses of MR vaccine is shown next to a 1 mL syringe.

4.3.2 Juvenile rhesus macaque vaccination with a microneedle patch

Juvenile rhesus macaques, approximately two years old, were vaccinated via MN patch or SC injection. Mild, transient erythema was noted for a few hours after MN patch administration; no adverse effects were noted at any point during the study. The humoral response was studied at day 0, 28, and 56 after vaccination. Vaccination with MN patch or SC injection induced measles neutralizing antibodies and measles-specific IgG titers; no statistically significance between the immune responses is noted (Figure 4.2).

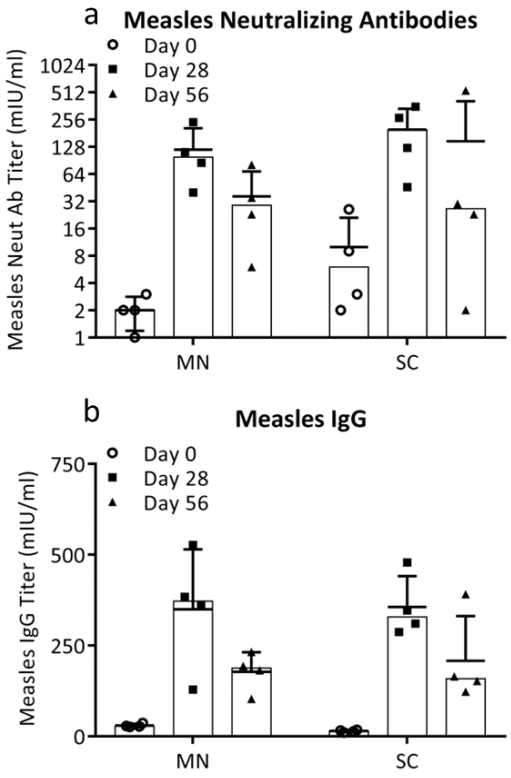

Figure 4.2. Juvenile rhesus macaque response to measles vaccination.
Measles antibody titers following vaccination of juvenile rhesus macaques with a microneedle (MN) patch or subcutaneous (SC) injection. Measles-specific neutralizing antibodies were measured on day 0 (circle), day 28 (square), and day 56 (triangle). The height of the bars is the median for the group, while the dash represents the mean +/- standard deviation. Vaccination by either vaccination route generated equivalent antibody titers.

Rubella neutralizing antibodies and rubella-specific IgG titers were determined at day 0, 28, and 56 post-vaccination (Figure 4.3). All titers increased after vaccination. At both post-vaccination time point, all juvenile rhesus macaques had protective neutralizing antibody titers of ≥10 IU/ml. The antibody titers were equivalent between the MN patch and SC injection group. Thus, MR can safely be delivered in a MN patch; MN patch vaccination induces a comparable immune response to SC injection.

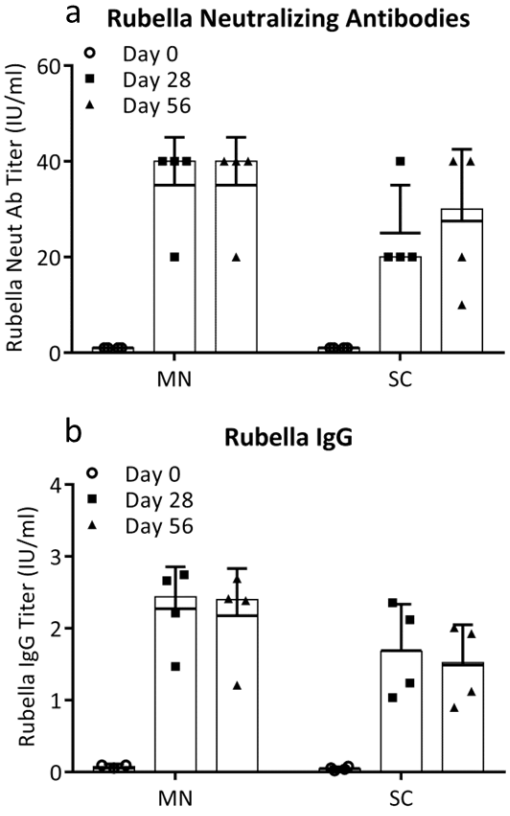

Figure 4.3. Juvenile rhesus macaque response to rubella vaccination.
Rubella antibody titers following vaccination of juvenile rhesus macaques with a microneedle (MN) patch or subcutaneous (SC) injection. (a) Rubella-specific neutralizing antibodies and (b) rubella-specific IgG were measured on day 0 (circle), day 28 (square), and day 56 (triangle). The height of the bars is the median for the group, while the dash represents the mean +/- standard deviation. All juvenile macaques had neutralizing antibody titers above the protective threshold (≥10 IU/ml). Vaccination by either vaccination route generated equivalent antibody titers.

4.3.3 Vaccination with a microneedle patch in infant rhesus macaques

Rhesus macaques, born to measles-naïve mothers, were vaccinated at 3-4 weeks of age (Table 4). MN patches were manually applied to the inner thigh of the macaques and left in place for 15 min to allow dissolution (Figure 4.4a). Immediately after removal, an array of puncture marks corresponding to the sites of MN puncture were detected on the skin (Figure 4.4b), but they were not visible 1 hour after vaccination. Additionally, only very mild, transient erythema and no edema or bleeding were observed; no adverse effects were noted at any point during the study.

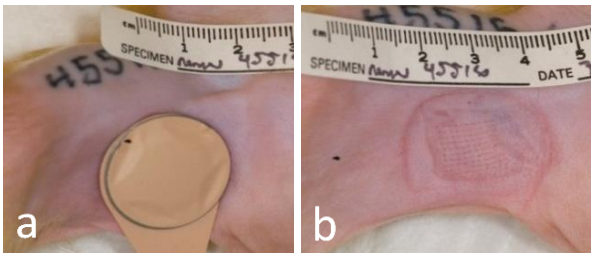

Figure 4.4. Microneedle patches inserted into infant rhesus macaque.
Microneedle patches (a) were applied to the inner thigh of infant rhesus macaques. (b) Immediately after removal, a grid pattern can be seen on the leg due to tiny punctures from each microneedle. After a few minutes, this grid pattern disappeared (not shown). Very mild, transient erythema, but no edema or bleeding were observed following removal of the patch.

In the groups that did not receive MiG (Figure 4.5, Table 4), the titers of neutralizing antibody to measles increased after vaccination, and at day 42, 100% of infant rhesus macaques in the MN patch group had protective titers of >120 mIU/ml, while 50% of rhesus macaques in the SC group had protective titers. On the day of challenge, all of the macaques in the MN group and 75% of the macaques in the SC group had protective titers (data not shown). The failure of one of the macaques in the SC group to seroconvert is unclear though previous studies have shown 75-80% of rhesus macaques vaccinated by

the SC route generated protective titers [82, 85]. Overall, these data indicate that vaccination by MN patch induced a neutralizing antibody response that was at least equivalent to the response induced by SC injection in naïve infant macaques.

In contrast, infant macaques vaccinated by MN patch or SC injection after receiving MiG failed to generate protective neutralizing antibody responses to measles by day 42 (Figure 4.5, Table S1). In these groups, the input MiG was detected by both PRN (Figure 4.5) and EIA (data not shown) on day 42. Previous studies showed that MiG can be detected in infant macaques for up to 8-10 weeks after administration [82, 84]. All of the macaques in the MiG treatment group were seronegative for measles as tested by PRN and EIA on the day of challenge approximately 30 weeks after MiG administration (data not shown).

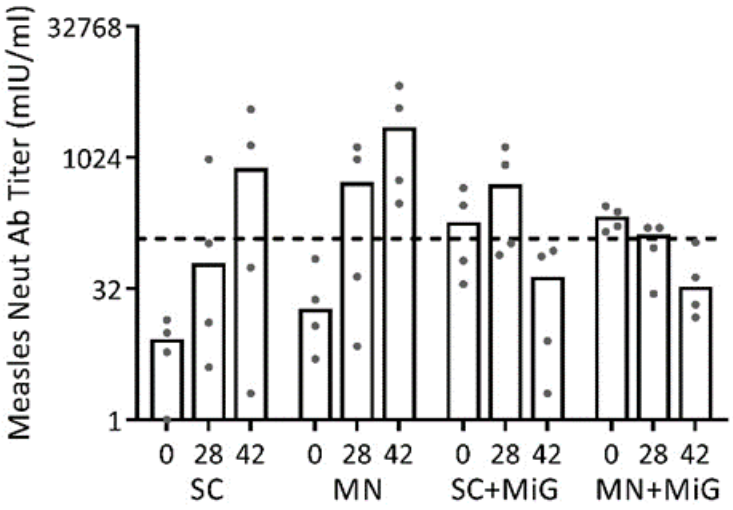

Figure 4.5. Measles neutralizing antibody titers.
Measles antibody titers following vaccination of infant rhesus macaques with a microneedle (MN) patch or subcutaneous (SC) injection in the absence or presence of measles immune globulin (SC+MIG, MN+MIG). Measles-specific neutralizing antibodies were measured on day 0, day 28, and day 42. The bars represent the mean neutralizing antibody titer of each group; dots represent titers for each rhesus macaque. The dotted line indicates the minimum protective titer of 120 mIU/ml. Protective titers of neutralizing antibodies were present in the MN and SC groups; vaccination by either vaccination route was unable to generate protective antibody titers after pre-administration of MiG.

The presence of measles-specific MiG had no effect on rubella vaccination. In both the presence and absence of MiG, all of the infant macaques had protective titers of neutralizing antibodies to rubella on day 42 (Figure 4.6a). Additionally, all titers were substantially above a protective titer of 10 IU/ml. Rubella IgG titers were equivalent across all groups at day 28 following vaccination (Figure 4.6b). In general, rubella neutralizing antibody titers were higher after MN patch vaccination compared to SC injection, but these differences were not significant. Overall, these data show that MiG inhibited the generation of an immune response to measles vaccines given by the SC or intradermal routes and that rubella vaccination by MN patch or SC injection induced robust neutralizing antibody responses.

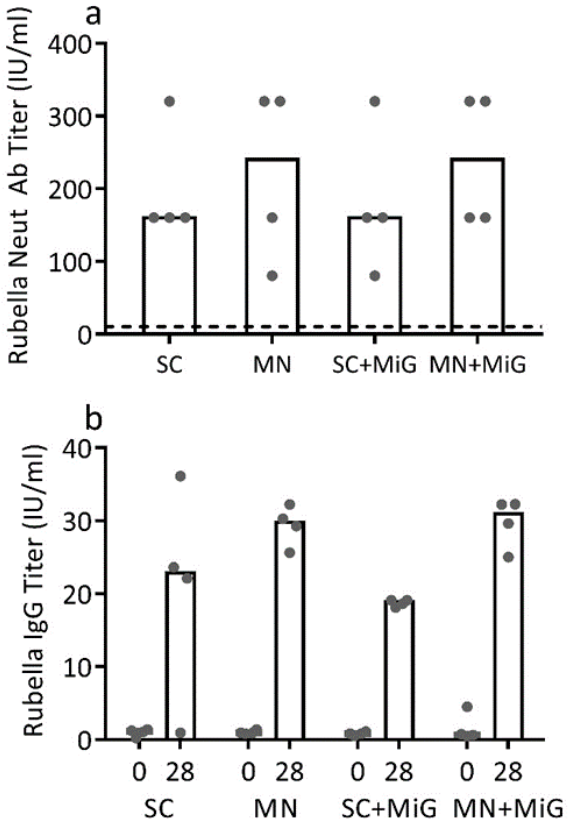

Figure 4.6. Rubella antibody titers.

Rubella antibody titers following vaccination of infant rhesus macaques with a microneedle (MN) patch or by subcutaneous (SC) injection in the absence or presence of measles immune globulin (MN+MiG, SC+MiG). (a) Rubella-specific neutralizing antibodies were measured on day 42. The bars represent the mean neutralizing antibody titer of each group; dots represent titers for each rhesus macaque. Dashed line indicates the protective titer of 10 IU/ml. (b) Rubella-specific IgG antibodies were measured on day 0 and day 28. Protective titers of IgG were present in the SC, MN, MN+MiG, and SC+MiG groups. The presence of MiG did not affect the response to rubella vaccine. Circles indicate titer for each macaque, while the bars indicate the mean within the group.

4.3.4 Measles challenge

Approximately seven months after vaccination, all groups, as well as four unimmunized controls, were challenged intranasally with wild-type measles virus. All of the infant macaques vaccinated in the absence MiG showed no clinical signs of infection such as coughing or rash, except for the one macaque in the SC group that failed to seroconvert (data not shown). Infant macaques vaccinated with MN patches no detectable viremia as measured by detection of infectious measles virus or viral RNA in PBMCs 7 and 17 days after challenge (Figure 4.7). Other than the infant rhesus that failed to seroconvert, all macaques in the SC injection group had no detectable infectious measles virus in their PBMC, though a low level of measles RNA was detected in one macaque (Figure 4.7). Therefore, vaccination of infant macaques with a MN patch induced a protective immune response, which was at least comparable to the protection provided by SC injection.

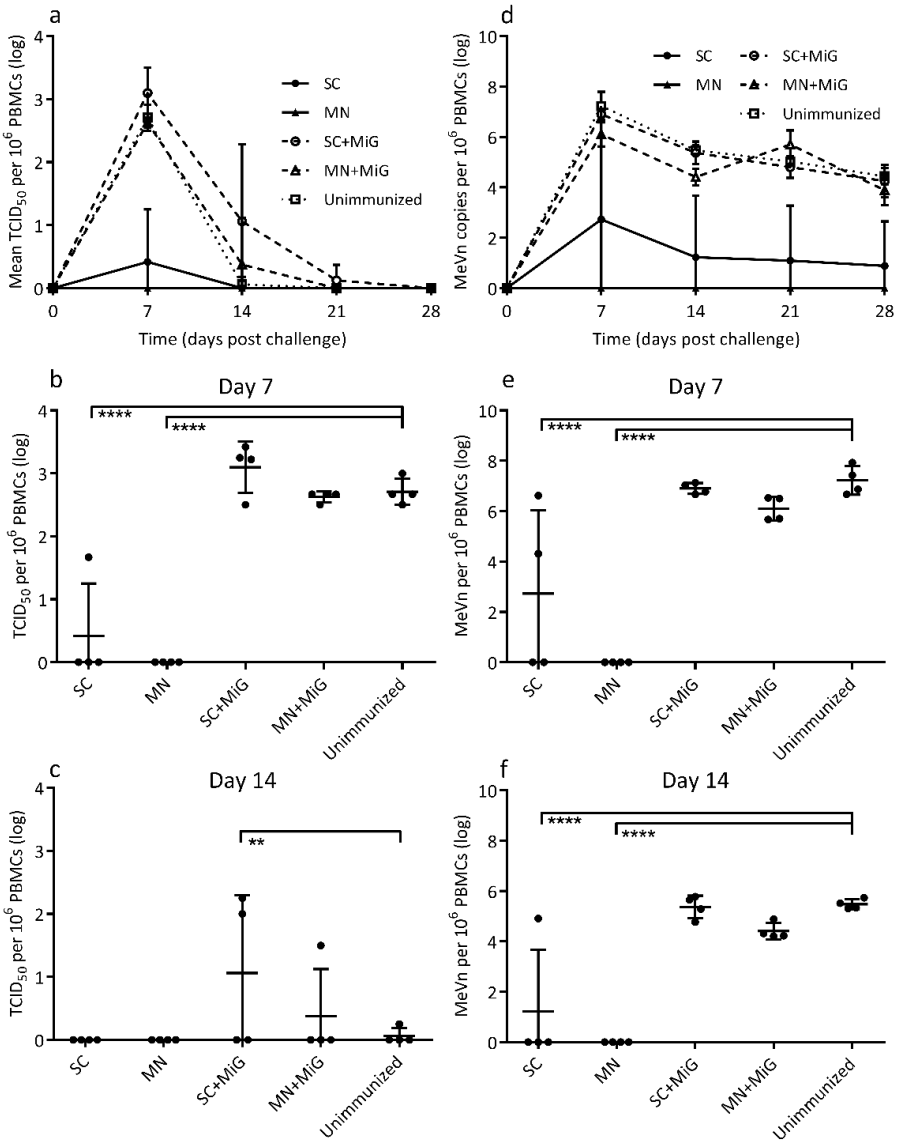

Figure 4.7. Measles response to challenge.
Vaccination with an MR vaccine by microneedle patch (MN) or by subcutaneous in (SC) protects infant rhesus macaques from challenge with wild type measles virus. At 202-216 days (mean 212 days, mean age 244 days) after vaccination, all vaccinated macaques and four naïve controls (means age 365 days, unimmunized) were challenged with 1 x 10^5 $TCID_{50}$ by the intranasal route. PBMCs were obtained from all macaques on the day of challenge (day 0), as well as 7, 14, and 21 days after challenge. Measles viremia was measured by co-culturing dilutions of PBMCs with Raji cells and extracting RNA from PBMCs and conducting RT-qPCR to determine the number of copies of measles RNA in each sample. Results are expressed as (a-c) mean log_{10} $TCID_{50}$ of measles virus or (d-f) mean log_{10} copies of measles RNA per 10^6 PBMCs. In the absence of MiG, all macaques vaccinated with the MN patch and three of four macaques vaccinated by SC injection were protected from challenge. Infant rhesus macaques that were vaccinated in the presence of measles immune

globulin (MN+MiG, SC+MiG) and one macaque in the SC group that did not have a protective titer were not protected from challenge.

All eight infant rhesus vaccinated in the presence of MiG displayed clinical signs of infection which were comparable to those of the unimmunized controls (data not shown). All of the macaques had viremia as measured by detection of infectious measles virus and measles RNA in PBMCs at days 7 and 14 after challenge. The viral titers and copy number of measles RNA in macaques vaccinated in the presence of MiG were similar to those of the unimmunized controls (Figure 4.7).

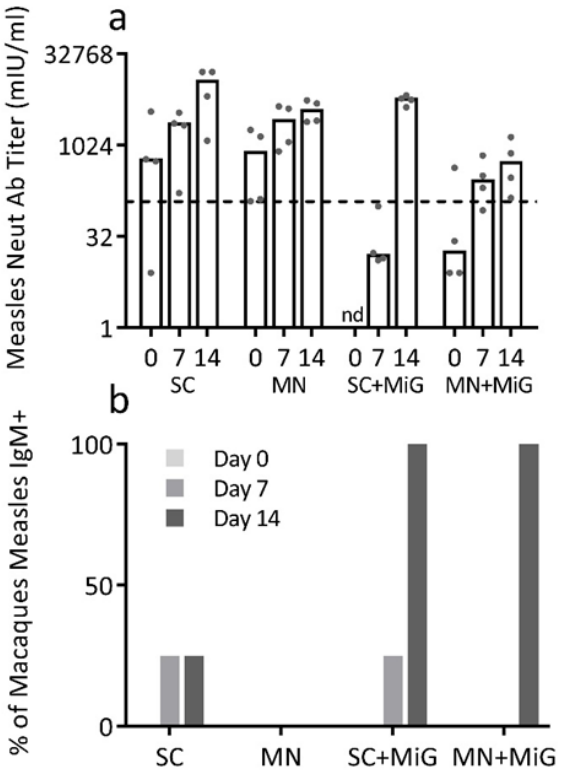

Figure 4.8. Humoral response to challenge.
Serologic response to measles virus following challenge with wild-type measles virus in infant rhesus macaques previously vaccinated against measles and rubella with a microneedle (MN) patch or by subcutaneous (SC) injection. All vaccinated macaques were challenged with 1 x 10^5 $TCID_{50}$ of measles virus by the intranasal route 202–216 days (mean 212 days, mean age 244 days) after vaccination. (a) Means measles-specific neutralizing antibody titers are shown as bars and titers for each rhesus are shown as dots. The horizontal dashed line indicates a protective titer of 120 mIU/ml.

(b) Measles-specific IgM titers were measured on the day of challenge (day 0, light gray bar), as well as day 7 (medium grey bar) and day 14 (dark grey bar) days after challenge. All macaques in the MiG treatment group seroconverted to measles by day 14, and all had an IgM response.

In the macaques vaccinated in the presence of MiG, protective neutralizing antibody titers were detected in all of the macaques by day 14 after challenge, and the titers of neutralizing antibodies boosted in all the macaques vaccinated in the absence of MiG (Figure 4.8a). With the exception of the macaque from the SC group that failed to seroconvert to vaccination, there was no measurable IgM following challenge in the macaques vaccinated in the absence of MiG (Figure 4.8b). However, all of the macaques vaccinated in the presence of MiG had an IgM response by day 14 after challenge suggestive of a primary immune response to measles (Figure 4.8b).

4.4 Discussion

4.4.1 MR vaccination by microneedle patch is immunogenic in rhesus macaques

In this study, we developed MN patches that administer both measles and rubella vaccines in a single dose. Juvenile rhesus macaques generated virus specific antibodies to both vaccines. The antibody titers were equivalent those receiving a SC injection. In naïve infant rhesus macaques, vaccination via MN patches induced a potent immune response to both measles and rubella vaccines. Measles- and rubella-specific neutralizing antibodies were well above protective titers of 120 mIU/ml and 10 IU/ml, respectively. Additionally, virus-specific IgG titers after MN patch vaccination were statistically indistinguishable from those of monkeys vaccinated via SC injection. Following challenge with live measles virus, vaccinated animals had strong neutralizing antibody titers rapidly boosted by the viral challenge (without generation of IgM) and showed no clinical signs of infection (e.g.,

rash), viremia or presence of measles viral copies. These data combined indicate that MN patches can effectively vaccinate infants and induce an equivalent protective immune response to traditional needle and syringe injection.

4.4.2 Microneedle patch can improve vaccination coverage

Improved MR vaccination coverage is crucial to meeting elimination goals. The traditional needle-and-syringe method for delivering vaccinations faces numerous challenges, particularly when used for MR elimination and future eradication efforts involving mass vaccination, ideally conducted house-to-house [124]. First, current vaccines are available in multi-dose lyophilized vials which necessitate storage in the cold chain, reconstitution before injection, and discarding vaccine if not used within 6 hours of reconstitution [32]. The vaccine is then injected SC by a trained health care provider, who must properly dispose of the hypodermic needle sharps waste to avoid re-stick injuries and disease transmission. An alternative vaccination method could increase vaccination coverage by easing the logistics and reducing costs for vaccinations [126, 171].

In this work, we developed a MN patch designed to overcome many of the logistical barriers associated with traditional MR vaccination. MN patches have a single-dose presentation, which reduces vaccine wastage associated with multi-dose vials. Because the patches are administered in a dry state, no vaccine reconstitution is needed, which avoids the expertise needed to reconstitute vaccine, eliminates errors associated with incorrect reconstitution and gets rid of the additional vials and syringes needed for the reconstitution process. Also, because MN patches require no water or syringes for injection, they have a much smaller packaged volume and weight, thereby facilitating transportation, storage and

waste disposal, which can be important in mass vaccination campaigns, especially when involving access to remote locations.

Through improved formulation and fabrication, MN patches can be stored at elevated temperatures for at least one month without loss of activity, thereby saving on refrigeration costs and reducing vaccine wastage associated with cold-chain failures [158]. Because MN patches are simple to administer, they can be used for MR vaccination by minimally trained personnel, an attribute that has been critical to smallpox and polio eradication programs in developing countries where expert health care personnel are in short supply. Finally, MN patches are expected to be inexpensive to manufacture and have been shown to be effective for delivery of a variety of vaccines [5, 26, 158-160], including prior studies of measles vaccination in cotton rats and juvenile rhesus macaques [20, 88] and a recent human clinical trial of influenza vaccination [16].

Jet injectors, which utilize a stream of liquid at a high pressure to pierce through skin, is a needle-free vaccination method that has attracted interest because it does not generate biohazardous sharps waste; however, the administration of these injectors requires training and offers no benefits for thermostability. In adults and toddlers, measles-mumps-rubella (MMR) vaccination with a jet injector induced an immune response comparable to traditional needle-and-syringe injection, but had significantly higher pain scores [58, 59]. However, in another MMR vaccination study, the jet injector induced inferior measles and mumps antibody titers but equivalent rubella antibody titers compared to SC injection in a clinical trial in infants [60]. The low measles titers were attributed to incomplete delivery when using the jet injector.

Another alterative mode of measles vaccination involves delivery of aerosolized vaccine by inhalation [64, 67]. While inhaled measles vaccine in schoolchildren compared favorably to SC injection in one study, another study in infants showed inferior immune responses compared to SC injection [64, 67]. Clinical studies in adults have demonstrated the safety of aerosol vaccination [65, 66]. The immune response was comparable to SC injection; however, because all subjects were measles positive initially, the immune response in a naïve patient was unclear.

There has been limited prior attention to measles and rubella vaccination in the skin, other than our work on MN patch vaccination [20, 88]. One study utilized tape stripping to remove the outer layers of the skin followed by topical inoculation with measles vaccine. However, the serology showed an inferior response compared to a SC injection [57].

4.4.3 Maternal antibodies hinder vaccination

The presence of maternal antibodies hinders efforts to vaccinate infants early in life; this window of susceptibility accounts for approximately 20% of cases worldwide [163]. Maternal antibodies wane between 6 to 9 months of age; infants vaccinated during this time seroconvert 60% of the time, compared to 95% of infants vaccinated between 12 and 15 months [81, 82]. Previous work has studied approaches to allow for earlier vaccination in the presence of maternal antibodies. For example, DNA vaccines were only able to produce a protective response when co-administered with IL-2 molecular adjuvant [85, 87]. However, use of this adjuvant may raise safety concerns. Here, we hypothesized that the heightened immune responses sometimes associated with skin vaccination could enable

measles vaccination via a MN patch to overcome the inhibitory effects of MiG. However, the resulting immune response was not strong enough to protect these animals from challenge. More work to develop more potent vaccines, adjuvants and delivery methods will be necessary to enable vaccination closer to the time of birth.

4.5 Conclusion

Measles and rubella control programs need higher levels of vaccination rates to achieve elimination goals. At the 2016 Global Vaccine and Immunization Research forum, public health officials emphasized the critical need for novel vaccine delivery tools, and that these tools could represent a "potential game-changer" for eradication efforts [133, 171]. MN patches may provide a MR vaccination method that addresses these needs; however, in previous MN patch studies, MR vaccination and thermostabilization have not been studied before, administration of any vaccine to infant primates has not been investigated, and the ability of skin vaccination by MN patch to overcome MiG has not been assessed.

To address these needs, this study developed MN patches for MR vaccination with attributes designed to offer significant benefit over traditional needle-and-syringe delivery techniques, especially for vaccination campaigns. The MN patches have a single-dose presentation requiring no reconstitution, which should reduce vaccine wastage, and enabling at least partial removal from the cold chain. They are simple to administer and generate no sharps waste, suggesting their suitability for administration by minimally trained personnel. Their small size and weight, in addition to expected low cost of manufacturing, are additional attributes of interest. In animal studies, the MN patches were

well-tolerated in juvenile and infant rhesus macaques; only mild, transient effects were seen at the MN patch application site and no long-term adverse effects over the course of the study were noted. Immunologically, MR vaccination by MN patch in infant macaques was not significantly different from SC injection of the vaccine and provided strong neutralizing antibody responses that protected animals from live measles virus challenge. However, MN patch vaccination could not generate protective immune responses in monkeys with MiG to simulate maternal antibodies. Overall, we conclude that MR vaccination using MN patches offers superior attributes important to mass vaccination with similar immunological outcomes. In this way, MN patch vaccination can enable increased vaccination coverage that is critical to MR elimination efforts.

CHAPTER 5. EXTENDED DELIVERY OF VACCINES TO THE SKIN IMPROVES IMMUNE RESPONSE

Vaccines prevent 2-3 million childhood deaths annually; however, low vaccine efficacy and the resulting need for booster doses creates gaps in immunization coverage. Here, we explore the benefits of extended antigen release into skin to increase immune responses after a single dose. By administering daily intradermal injections of inactivated polio vaccine according to six different profiles, zeroth-order release over 28 days resulted in neutralizing antibody titers equivalent to two bolus vaccinations administered one month apart. Vaccinations following this profile also improved immune responses to tetanus toxoid and subunit influenza vaccine but not measles vaccine, which naturally persists in the body as a live-attenuated virus. Finally, using subunit influenza vaccine, we demonstrated that daily vaccination by microneedle patch induced a potent, balanced humoral immunity with an increased memory response compared to bolus vaccination. We conclude that extended presentation of antigen in skin via intradermal injection or microneedle patch can enhance immune responses and reduce the number of vaccine doses, thereby enabling increased vaccination coverage.

5.1 Introduction

Vaccines are lauded as one of the top ten public health interventions of the past century [1], with immunizations resulting in the prevention of 2-3 million childhood deaths annually [172]. However, an additional 1.5 million deaths occur due to lack of adequate vaccination coverage [172]. In developing countries, vaccinations are often provided by

mass vaccine campaigns that include fixed clinics as well as trained personnel traveling house-to-house to reach remote areas [26]. Despite these efforts, inherent properties of vaccines complicate the ability to deliver doses at the appropriate time intervals. Additionally, low seroprotection rates require multiple costly, resource intensive vaccinations to limit the spread of disease [39]. Children who are immunocompromised by inadequate nutrition and co-existing infections seroconvert at rates lower than would be expected [26, 173, 174]. Furthermore, the majority of vaccines recommended by the WHO Expanded Programme on Immunization are delivered by hypodermic needle and syringe [175]. This creates a requirement of trained healthcare providers to deliver injections and dispose of the needles safely to prevent transmission of blood-borne pathogens. Revamping vaccine technology to increase vaccine immunogenicity, reduce the number of vaccinations and simplify the vaccination method could lead to increases in immunization coverage.

One strategy to increase the immune response to vaccination is prolonging antigen presentation [39]. There are two major categories of vaccines: live-attenuated vaccines and killed/subunit vaccines. The former, such as measles, smallpox, and varicella, mimic immunity that results from infection and can provide lifelong protection after a single administration. However, the majority of vaccines belong to the latter group and require one or more booster administrations separated by months or years to produce lasting immunity [176]. Extended delivery of an antigen can mimic a natural infection's kinetics, thereby generating a much stronger immune response to the same dose. However, common approaches to utilize controlled release systems follow kinetics with a large burst, followed by low levels of antigen release. While these systems do improve the immune response,

other profiles may allow for a stronger response. Johansen et al. delivered gp33 and CpG to vaccinate against lymphocytic choriomeningitis virus. They compared exponentially increasing, exponentially decreasing, constant, and bolus injections over 4 days delivered by daily injection. They report significantly higher levels of CD8+ active T cells in the exponentially increasing group, likely due to the mimicked kinetics to a replicating pathogen [177]. In an *in vitro* study, dendritic cells released higher levels of IL-2 and IL-10 cytokines when stimulated in an exponentially increasing fashion [177]. Other studies have replicated this finding and demonstrated increased antigen capture and retention in lymph nodes is responsible for the increased antibody response [109, 113]. Thus, the literature suggests that extended presentation of some model antigens can increase immune responses. However, studies are needed that use licensed vaccines, conduct a detailed examination of antigen delivery profiles, and determine if a single dose of vaccine delivered over an extended time can provide protective immunity comparable to that after a two-dose prime-boost schedule.

Intradermal (ID) delivery can improve immune responses. The skin, unlike muscle and subcutaneous space, is an immunological organ with roles in both innate and adaptive immunity [26]. The epidermis is largely composed of keratinocytes, expressing a variety of receptors specialized in recognizing pathogen-associated molecular patterns and bacterial, fungal, and viral antigens [178]. Antigen-presenting cells, such as epidermal Langerhans cells and dermal dendritic cells, can effectively capture, process, and present antigens. APCs can then travel to nearby lymph nodes, activating the adaptive immune response [179, 180]. Studies have demonstrated more efficient antigen migration into draining lymph nodes than intramuscular injections [181]. Additionally, higher levels of

activated T follicular helper cells (TFH) and germinal center B cells (GC B) were identified in lymph nodes following skin vaccination [45]. By delivering antigen directly to the skin, intradermal vaccination enhances immunogenicity, and can thereby reduce the number of doses needed to seroconvert or result in significant dose-sparing of vaccines. This effect was seen in several different applications, including smallpox [182], rabies [183], and influenza [184].

While ID vaccination offers improvements to the immune response, delivering antigen into the skin is difficult. The Mantoux technique, in which the needle is inserted at a shallow angle, requires special training to reliably target the skin [185]. Dissolving microneedle (MN) patches are an emerging technology for ID vaccinations [5, 28, 158-160]. MN patches are an array of polymer needles that are less than 1 mm long and fixed into a hard backing. The patch can then be painlessly applied to the skin, delivering vaccine antigen in a controlled fashion directly into the epidermis and dermis [19, 115, 159, 186]. Once the needles dissolve, the needles cannot be reused, and the backing can be discarded. Vaccination with MN patches have induced potent immune responses, even demonstrating dose-sparing in some cases [41, 43, 44]. Extended release via microneedles has also been shown [14, 115, 116]. Patches of silk-tipped MNs delivering whole-protein vaccine released over 1-2 weeks resulted in over a tenfold increase in adaptive immune response compared to subcutaneous or intramuscular bolus injections [187]. The use of MN patches for extended delivery of vaccines has received only limited attention.

Guided by these studies, we envision a vaccine delivery system that targets antigen to the skin and presents that antigen over an extended period of time to improve immunogenicity, using a MN patch that simplifies vaccination. Here, we examined the

effect of six different extended delivery profiles on immunogenicity using four different licensed vaccines administered to the skin. Extended delivery profiles were studied by administering multiple fractional vaccine doses given by intradermal injection or microneedle patch. In this context, we compared single doses of extended delivery vaccination to two-dose bolus vaccination. We showed for the first time that extended delivery for one month further improves the immune response compared to extended delivery over shorter periods of time. Additionally, extended delivery achieved through repeated dissolving MN patch vaccination further increases the immune response compared to injections. Future studies will address development of formulations that can achieve the optimized delivery profiles after application of a single MN patch.

5.2 Materials and methods

5.2.1 Vaccines

Monovalent stock solutions of types 1, 2 and 3 IPV vaccine were generously provided by GlaxoSmithKline Biologics (Rixensart, Belgium). These vaccine antigens are the same as those used in Poliorix. Influenza subunit vaccine bulk (A/Brisbane/10/10) was generously provided by Seqirus (Maidenhead, United Kingdom). This vaccine antigen is the same as that used in the 2015-2016 seasonal vaccine Fluvirin. Tetanus toxoid vaccine bulk was kindly supplied by the Serum Institute of India (Pune, India). This vaccine antigen is the same as that used in Tetanus Toxoid Vaccine Adsorbed. The Edmonston-Zagreb strain of measles virus vaccine (kindly supplied by the Serum Institute of India) was grown in Vero cells maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) and 2% fetal bovine serum (FBS, Gibco). At the maximum of the cytopathic

effect, cell suspensions were freeze-thawed and centrifuged to eliminate cellular debris, as previously described [20]. This measles virus is the same as that used in M-VAC, but propagated using Vero cells at CDC rather than in MRC-5 cells at the Serum Institute of India. All vaccines were diluted in sterile saline solutions prior to injections.

5.2.2 Microneedle patch

MN patches were fabricated as described previously [9, 167]. Briefly, influenza vaccine was mixed with 10% w/v sucrose (Sigma Aldrich, St. Louis, MO) and 1% w/v sodium carboxymethyl cellulose (250 kDa, Sigma Aldrich) in deionized water. This solution was cast onto silicone molds under vacuum and dried. A polymer matrix solution of 28% w/w polyvinyl alcohol (78% hydrolyzed, 6 kDa; Acros Organics, Geel, Belgium), 21% w/w sucrose, and water was added to the mold, dried. MN patches were removed from their molds and stored at room temperature (22-25°C) with desiccant until use for vaccination. Placebo patches were fabricated with only the polymer matrix solution.

5.2.3 Animal studies

On vaccination days, rodents were anesthetized either daily for seven days or every other day for fourteen or twenty-eight days. Anesthesia was induced with 5% isoflurane and maintained during the procedure at 2%. Animals were then vaccinated by injection in the skin using a 28 gauge hypodermic needle by the Mantoux method, in the muscle using a 28 gauge hypodermic needles, or by MN patch applied to the skin. All injections administered 20 or 100 µL of vaccine containing full or fractional vaccine doses such that the cumulative dose received by all animals was the same, as described for each experiment. Animals receiving booster doses received a second full dose, such that those

animals received a cumulative dose twice that of the non-boosted groups. All doses were verified by enzyme-linked immunosorbent assay (ELISA) or end-point titration, in the case of measles vaccine [9, 71, 188]. Blood was collected at set time points; serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) were used to separate sera during centrifugation. All animal studies were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee (IACUC), and IPV vaccination studies were also approved by the CDC IACUC.

5.2.3.1 Inactivated polio vaccine

For IPV studies, female Wistar rats (Charles River, Wilmington, MA), age 6-8 weeks, were anesthetized with isoflurane (n=10 per group). Vaccine was diluted in sterile saline to the proper concentration. Rats were vaccinated with 100 ul either intradermally on the flank or intramuscularly in the thigh. In monovalent IPV studies, all rats received IPV type 2 at a cumulative dose of 0.8 D-antigen units (DU). In trivalent IPV studies, rats received 10 DU of type 1 IPV, 2 DU of type 2 IPV and 8 DU of type 3 IPV (which is 25% of the conventional IPV dose administered to humans [189]) via 100 ul injections. Blood was collected from the tail vein via laceration biweekly until day 56 or 84; for the longitudinal study, blood draws continued monthly until month 6.

5.2.3.2 Tetanus toxoid vaccine

Female Balb/c mice (Charles River, age 6-8 weeks) were vaccinated with tetanus toxoid vaccine intradermally at the base of the tail (n = 9 - 10 per group). The cumulative dose was 5 flocculation units (Lf) (which is equal to the conventional tetanus toxoid dose administered to humans [189]), administered either as a full-dose bolus (in 20 uL) or by

1/14th dose (in 20 uL) every other day over 28 days. Blood was collected via the jugular vein on days 0, 14, 28, and 42.

5.2.3.3 Measles vaccine

Female cotton rats (Harlan, Indianapolis, IN, age 8 weeks) were vaccinated with live-attenuated measles vaccine intradermally on the side (n= 10 per group). The total dose was 200 50% tissue culture infective dose ($TCID_{50}$) (which is 20% of the minimum conventional measles vaccine administered to humans [20]), administered either as a full-dose bolus (in 20 uL) or by 1/14th dose (in 20 uL) every other day over 28 days. Blood was collected via the jugular vein on days 0, 14, 28, 42, and 56.

5.2.3.4 Influenza vaccine

Female Balb/c mice (Charles River), 6-8 weeks of age, were immunized with influenza vaccine either via intradermal injection at the base of the tail, intramuscular injection in the thigh muscle or microneedle patch on the back (n=8 per group). A cumulative dose of 1 µg (which is 7% of the conventional influenza vaccine administered to humans [189]) was given either by full-dose bolus (in 20 uL) or by 1/14th dose (in 20 uL) every other day over 28 days. On day 77, groups that had received bolus vaccination were boosted with a second full-dose bolus. Blood was collected via the jugular vein on days 0, 14, 28, 42, 56, 77, 91, and 105.

In a second study, mice were vaccinated ID or by MN patch, receiving 1 ug total dose. Blood was collected on days 0, 7, 14, 21, 28, and 35. On day 28, the boost group

received another 1 ug by dose ID injection. On day 35, animals were euthanized; spleen, lymph nodes, and bone marrow were collected as previously described [190, 191].

5.2.4 IPV neutralizing antibody assay

Assay of poliovirus neutralizing antibodies was performed at the CDC Polio and Picornavirus Laboratory using their standard protocols [192] . Briefly, diluted serum samples were incubated with polioviruses types 1, 2, and 3 (Sabin) at 35°C for three hours prior to the addition to HEp-2(C) cells. After incubation for five days at 35°C, cells were stained with crystal violet and cell viability was measured by optical density. Titers were determined as the reciprocal of the dilution that was able to inhibit 50% of virus binding [188, 193]. Seropositivity was defined as antibody titers greater than or equal to 3.0.

5.2.5 Tetanus toxoid-specific antibody assay

Tetanus toxoid-specific IgG titers were determined via ELISA, as described previously [167] . Briefly, tetanus toxoid was coated on a microwell plate at 0.5 Lf/mL. Diluted serum samples followed by HRP- tagged anti-mouse antibody (Southern Biotechnology Associates, Birmingham, AL) were allowed to bind to the plate. Optical density values were compared to a standard curve of mouse IgG to determine titers.

5.2.6 Measles neutralizing antibody assay

Measles titers were determined using a plaque-reduction neutralization assay, as described previously [168]. Briefly, diluted serum samples were mixed with measles challenge virus (University of California, Davis, CA) and incubated for 2 h at 37°C. The samples were then plated onto 24-well plates with a confluent monolayer of Vero cells

(American Type Culture Collection, Manassas, VA) and incubated for an additional 2 h at 37°C, after which an overlay media of 0.8% CMC was added to the cells. Five to seven days later, media was removed, and crystal violet solution was added for 20 min. Plaques were counted in each well, and titers are determined according to the Third WHO International Standard Reference Serum (97/648).

5.2.7 Influenza hemagglutination assay

Hemagglutination inhibition (HAI) assay was used to determine influenza-specific antibodies as a correlate to immune response, as described previously [194]. Briefly, serum was treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) at 37°C overnight. The following day, samples were inactivated at 56°C for 30 min. Packed chicken red blood cells (Lampire Biological Laboratories, Pipersville, PA) were added and incubated with samples overnight. Light centrifugation was used to remove the red blood cells. Supernatant was serially diluted in phosphate-buffered saline (PBS). Samples were then mixed with A/California/07/2009 influenza virus for 30 min. Red blood cells were added and incubated for approximately 10 minutes until the titer at which agglutination was inhibited could be determined. The highest dilution titer that inhibited hemagglutination was read as the HAI titer. Seroprotection was defined as antibody titers greater than or equal to 40.

5.2.8 Influenza-specific antibody assay

Purified mouse IgG, IgG1, and IgG2a and goat anti-mouse-HRP were purchased from Southern Biotechnology Associates. Titers were determined via ELISA as previously described [83]. Briefly, subunit influenza vaccine was coated on microwell plate. Diluted

serum samples, horseradish peroxidase (HRP)-tagged antibody, and SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) were subsequently added to the plate. Once sufficient color had developed, reaction was stopped using TMB BlueSTOP Solution (KPL); plates were analyzed using absorbance at 620 nm.

5.2.9 Cellular analysis

5.2.9.1 Flow cytometry

Lymph nodes (LN, inguinal and axillary) were harvested from mice and single cell suspensions were prepared and stained with fluorescent anti-mouse antibodies for detection of germinal center forming B cells (GC B cell, B220+GL7+), T follicular helper (TFH, CD3+CD4+CXCR5+PD1+) and plasma blast (CD3-CD138+) cells by flow cytometry. For antibody staining, the cells were incubated with fluorescent anti-mouse antibodies in FACS buffer for 30 min at 4°C and washed with FACS buffer and fixed with BD Cytofix. Flow data was acquired using a BD LSRII flow cytometer and analysed by Flow Jo software.

5.2.9.2 ELISPOT assay

Subunit influenza vaccine specific antibody secreting plasma cells in bone marrow was detected by ELISPOT assay. For this, MultiScreen HTS-IP filter 96 well plate (Millipore, Billerica, MA) was coated with antigen (1 ug/ml) overnight at 4°C, and next day, one million bone marrow cells (collected from femur and tibia) without RBC in complete RPMI 1640 cell culture media was added to each well and incubated overnight in a 37°C incubator with 5% CO_2. Secreted anti-HA-IgG1 and IgG2a were then detected by alkaline phosphatase (AP) conjugated goat anti-mouse IgG1 and IgG2a antibodies

(Southern Biotech) and finally the antibody spots were developed by Vector Blue AP substrate (Vector Laboratories, Burlingame, CA). An ELISPOT reader was used to image and count the spots in the well.

5.2.9.3 Antigen restimulation studies

Antigen restimulation assay was performed to evaluate antigen specific Th1 immune response. For this, single cell suspension of splenocytes was first prepared by collagenase digestion of spleen and then one million splenocytes in complete RPMI 1640 media were incubated with antigen at 1 ug/ml concentration in a 96 well plate for 72 hrs. IFN gamma level in the restimulation media was determined by ELISA using a ready-set-go IFN gamma ELISA kit (EBioscience, San Diego, CA).

5.3 Results

5.3.1 Optimal delivery profile

Vaccines are conventionally administered as a bolus. Our initial studies were designed to identify the optimal release profile for vaccine delivered into the skin. In order to reduce the number of groups and therefore the number of animals, we separated the study into two parts: (1) to identify the optimal release profile and (2) to identify the optimal length of delivery. First, we studied six different delivery profiles of monovalent type 2 IPV administered over the course of seven days in order to understand the effect of the time course of antigen presentation, as seen in Figure 5.1. A bolus injection served as the control of standard vaccinations. Constant and Exponentially Increasing profiles mimic natural infection kinetics, where a pathogen persists and in some cases, multiplies over

time. An initial prime dose was followed by either a steady decrease in dose (Exponentially Decreasing profile) or a constant dose (High/Low profile) to provide a strong initial activation of the immune system followed by continued antigen presentation. Finally, the Rise/Fall profile provides a more gradual onset and offset of antigen presentation by using an exponential increase followed by a decrease in antigen dose over the course of a week. The cumulative vaccine dose administered in each of these profiles was held constant at 0.8 DU.

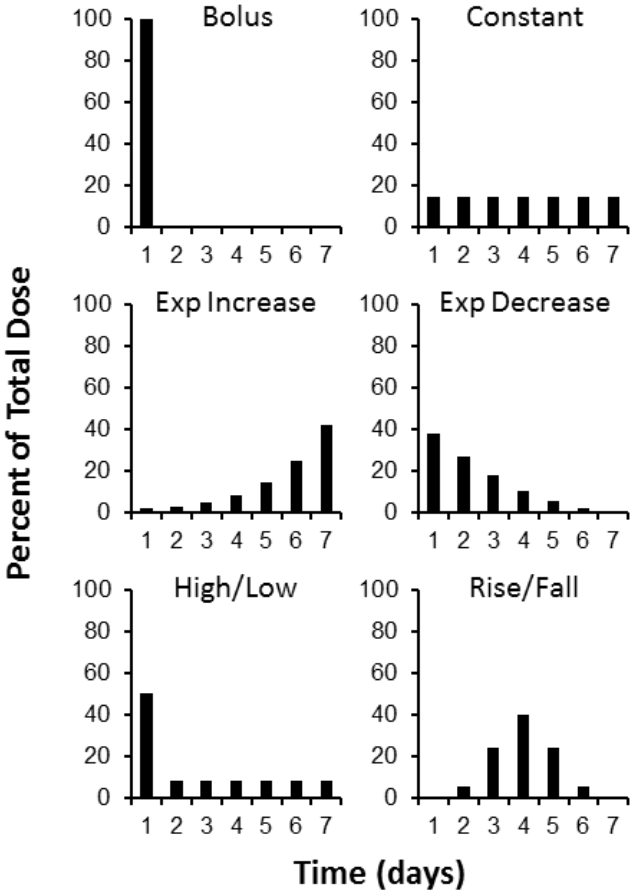

Figure 5.1. Kinetic profiles.
Daily intradermal injections were administered over seven days, following six different profiles.

Bolus delivery showed expected neutralizing antibody response over time, with an increase and plateau in titers over the course of eight weeks (Figure 5.2a) [195].

Exponential Decreasing, High/Low and Rise/Fall profiles did not yield significantly different antibody titers compared to bolus delivery ($p>0.90$). However, neutralizing antibody titers when the dosing followed Constant and Exponentially Increasing delivery were significantly greater than bolus delivery neutralizing antibody titers ($p=0.011$ and 0.005, respectively). This indicates that it was beneficial to prolong antigen delivery in a manner that maintained or increased dose over time, while profiles with an initial burst followed by low antigen levels were not beneficial.

Additional analysis of neutralizing antibody titers on Day 28 (Figure 5.2b) shows mean and median antibody titers that were 2-3 fold higher after Constant and Exponentially Increasing delivery compared to bolus delivery. Determination of seropositivity (Figure 5.2c) confirm these findings, showing 60 – 70% seropositivity after Constant and Exponentially Increasing delivery compared to only 40% seropositivity after bolus delivery. We selected the Constant release profile for further characterization. It appeared equivalent to the Exponentially Increasing profile with the added benefit of being easier to replicate in future studies. This profile could more easily be converted to a single-dose, controlled-release drug delivery system in the future.

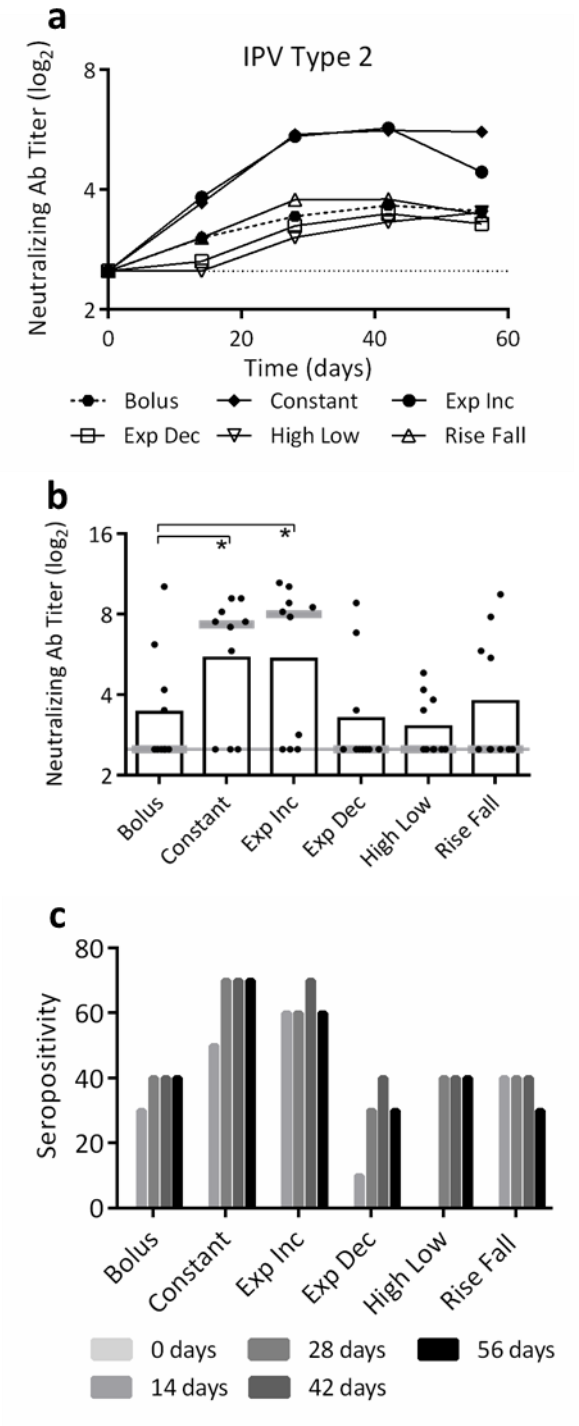

Figure 5.2. Release profile over seven days.
Neutralizing antibody titers over time (a) show constant and exponentially increasing profiles induce higher titers compared to bolus vaccination. (b) Titers at day 28 post initial vaccination; the dotted line at 2.5 indicated the assay baseline (*p<0.05). (c) Seropositivity is the percent of animals in each group with titers >3.0. Total dose was 10% of a human dose (0.8DU of IPV type 2).

5.3.2 Optimal length of delivery and boosting

Next, we investigated the effect of the total duration of delivery on the immune response, and more broadly assessed the effects of extended delivery by administering trivalent IPV (i.e., types 1, 2 and 3). Three experimental groups received a single dose distributed every day for 7 days, every-other day for 14 days, or every-other day for 28 days. These results were compared to a single bolus priming dose (Bolus-P) or a two-dose prime + boost regimen (Bolus-P+B). Immune response to type 2 IPV was similarly strong in all vaccination groups at day 84 following initial dosing (Figure 5.3b). In response to type 1 vaccination, 28-day exposure to antigen produced significantly better neutralizing antibody responses than a single priming dose and was statistically indistinguishable ($p=0.999$) from the two-dose prime + boost regimen (Figure 5.3a). Neutralizing antibody titers to type 3 IPV showed the greatest differences, where 7-day and 28-day extended vaccine delivery were superior to a single bolus dose ($p=0.035$ and <0.0001), and 28-day delivery was again statistically indistinguishable from the two-dose prime + boost regimen ($p=0.999$) (Figure 5.3c). Looking at the seropositivity data (Figure 5.4), only 28 days of constant dosing and the prime + boost group achieved 100% seropositivity across all three serotypes. This suggests that a single dose of a month-long slow-release formulation of IPV can produce immunity similar to a two bolus doses of vaccine.

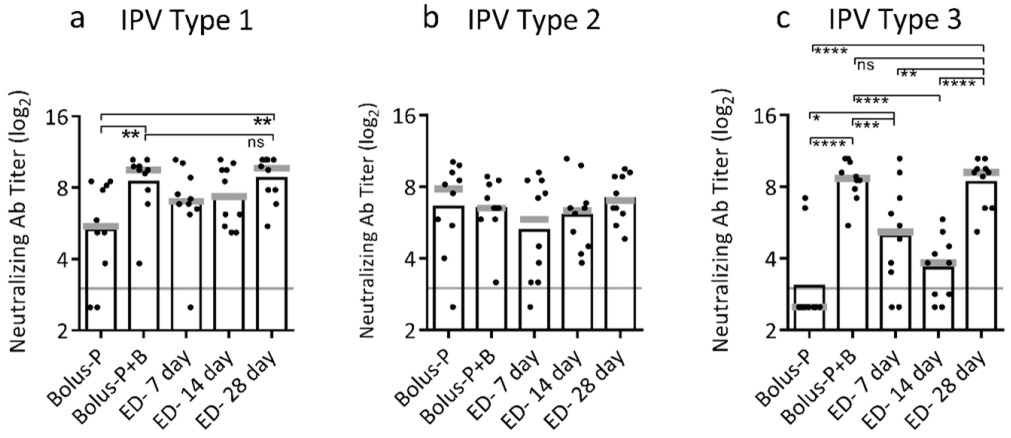

Figure 5.3. Duration of release- Titers at day 84.
IPV type one neutralizing antibodies at day 84 are represented in panel a; IPV type two in b; and IPV type three in c (ns- p>0.05; * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). These data show that extended delivery over 28 days induces neutralizing antibody titers equivalent to two doses administered one month apart. Total dose was 25% of a human dose (10 DU of IPV type 1, 2DU of IPV type 2, and 8 DU of IPV type 3).

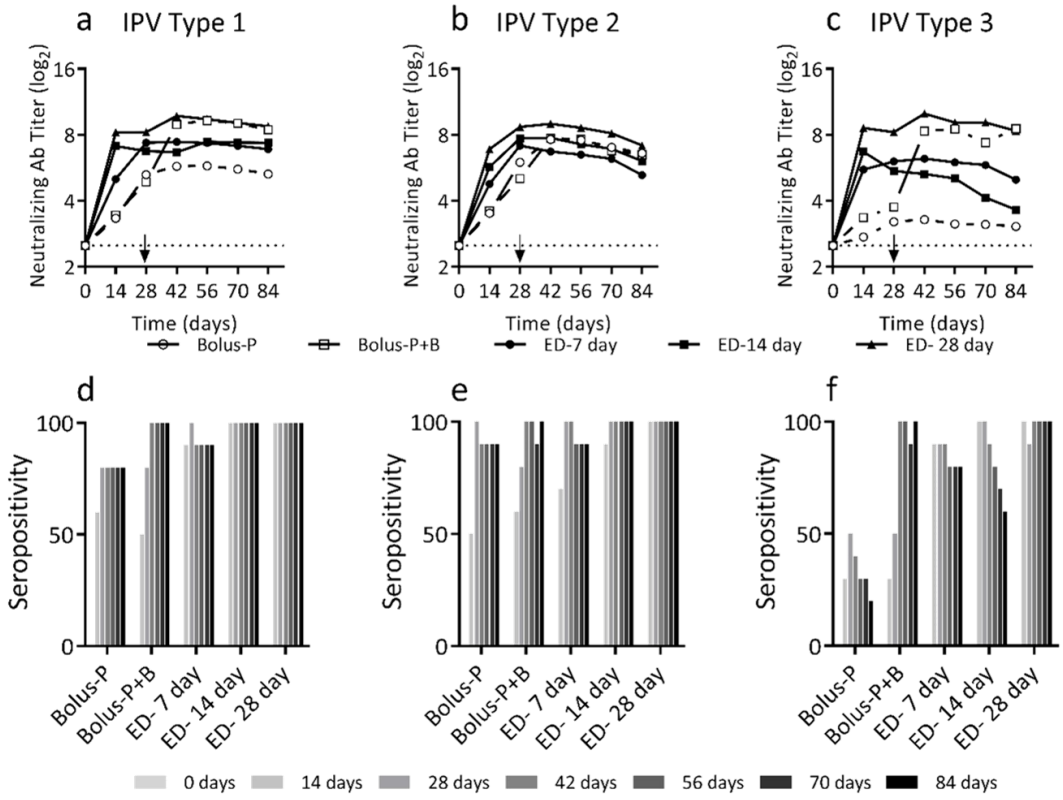

Figure 5.4. Duration of release- Titers over time and seropositivity.
IPV type one neutralizing antibodies are represented in panels a and d; IPV type two in b and e; and IPV type three in c and f. The top row of panels shows the titers over

time, with the arrow representing the boost on day 28 and the dotted line representing the baseline of the assay. The bottom row shows seropositivity at each time point.

To further understand the relationship between extended delivery and boosting, rats were administered trivalent IPV as a two-dose 7-day extended-delivery regimen to combine the benefits of extended delivery and boosting. This approach yielded neutralizing antibody titers greater than either a single bolus dose or a two-dose prime + boost regimen, which was statistically significant for types 2 and 3 IPV (p= 0.047 and 0.001) (Figure 5.5 and Figure 5.6). However, the two-dose extended-delivery regimen was not significantly different than a single 28-day extended delivery for any of the three serotypes. These data indicate that extended delivery can be used to improve either the prime or boost regime.

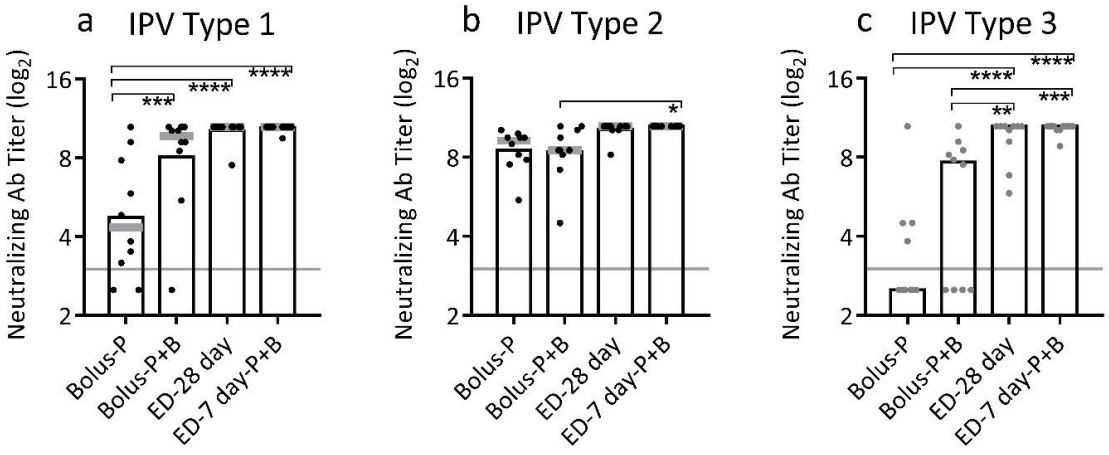

Figure 5.5. Boosting- Titers at day 56.
The titers at day 56 for each of the three isotypes of IPV are individually represented (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). Extended delivery over seven days during the boost increases immune response compared to bolus delivery for both doses. Total dose was 25% of a human dose (10 DU of IPV type 1, 2DU of IPV type 2, and 8 DU of IPV type 3).

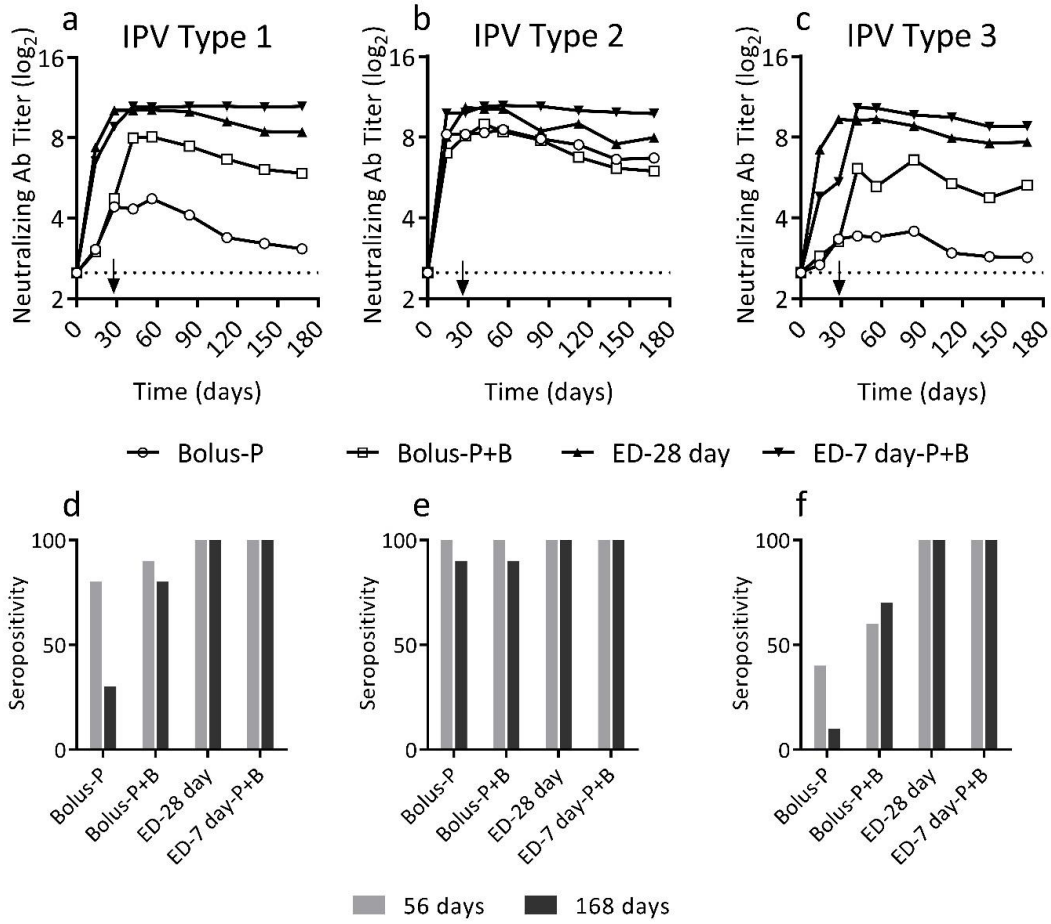

Figure 5.6. Boosting- Titers over time and seropositivity.
IPV type one neutralizing antibodies are represented in panels a and d; IPV type two in b and e; and IPV type three in c and f. The top row of panels shows the titers over time, with the arrow representing the boost on day 28. The bottom row shows seropositivity at day 56 and day 168 (6 months).

5.3.3 Effect of route of administration

To this point, we have identified optimal vaccine kinetics during intradermal vaccination. We next examined if the same profile would also be beneficial in the muscle. Rats were administered trivalent dose of IPV either intramuscularly (IM) or intradermally (ID) and either as a bolus or extended delivery over 28 days. Neutralizing titers against type 2 were equivalently high for all groups (Figure 5.7b, $p>0.999$). In response to type 1 and 3 vaccinations, there was no statistically significant difference in the immune response

between the bolus IM and bolus ID groups, nor was there a difference between the extended delivery of vaccine over 28 days delivered in the muscle or the skin (Figure 5.7a and c, p=0.86 and 0.95 for types 1 and 3, respectively). However, antibody titers in both IM and ID extended delivery groups were significantly higher than their bolus counterparts (Figure 5.7a and c, p<0.001). This indicates that extended delivery is beneficial after IM as well as ID vaccination. Furthermore, antibody titers remained high up to six months after vaccination. Extended delivery into the skin was the only group to maintain 100% seropositivity at six months after initial vaccination (Figure 5.8). This suggests that extended delivery vaccination induces long-lasting improvements in immune response.

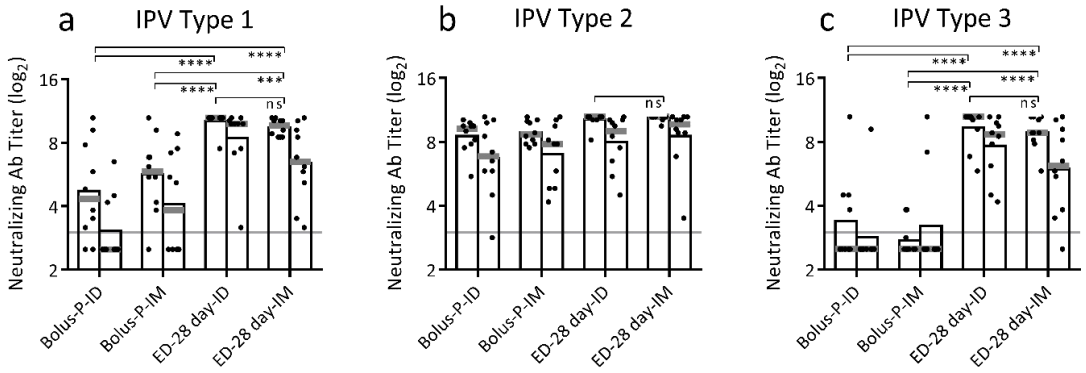

Figure 5.7. Route of delivery- Titers at day 56 and 168.
IPV neutralizing antibody titers for type 1 are shown in panel a, for type 2 are shown in panel b, and for type 3 are shown in panel c (ns- p>0.05; * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). Day 56 is represented by the left bar, and day 168 is the right bar. Extended delivery in the skin or muscle induces potent immune response compared to bolus vaccination with no difference between the two locations. Total dose was 25% of a human dose (10 DU of IPV type 1, 2DU of IPV type 2, and 8 DU of IPV type 3).

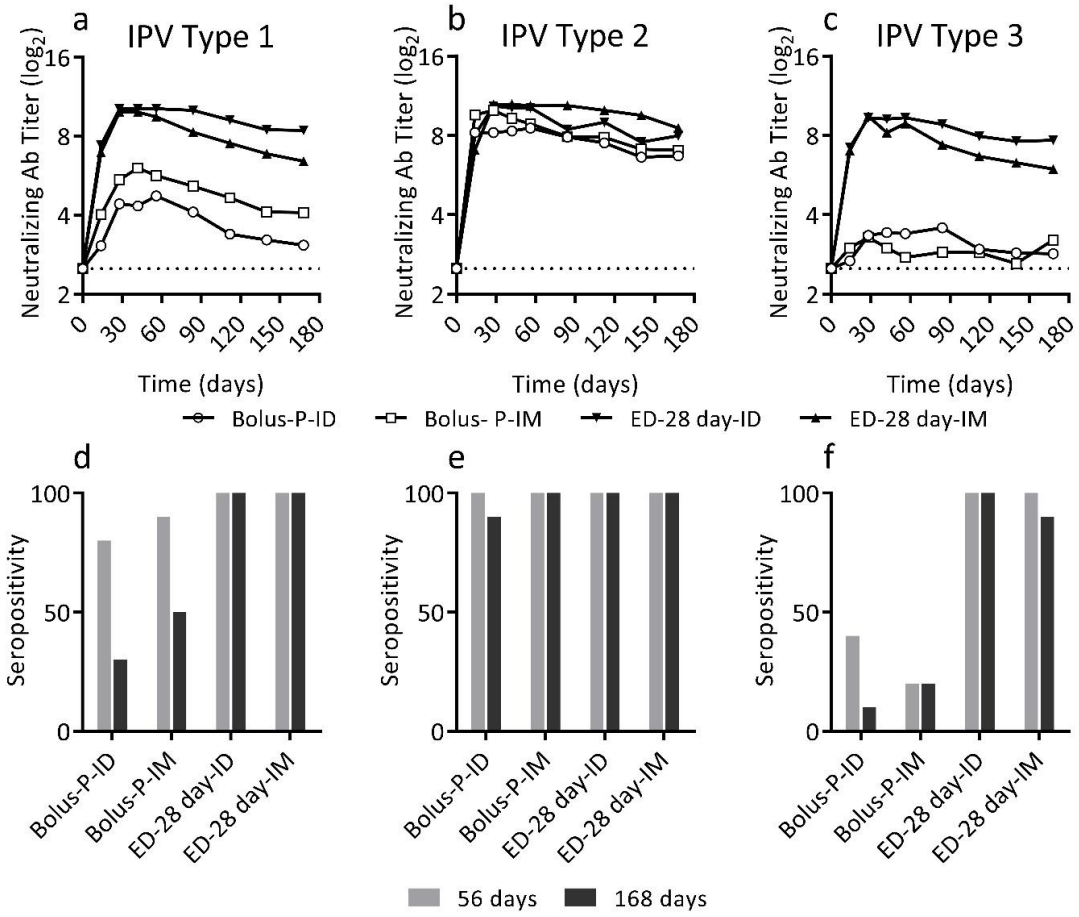

Figure 5.8. Route of delivery- Titers over time and seropositivity.
IPV type one neutralizing antibodies are represented in panels a and d; IPV type two in b and e; and IPV type three in c and f. The top row of panels shows the titers over time, with the arrow representing the boost on day 28 and the dotted line representing the baseline of the assay. The bottom row shows seropositivity at day 56 and day 168 (6 months).

5.3.4 Applicability to multiple vaccines

We next wanted to determine if the extended delivery profile we studied using IPV would broadly apply to other vaccines. To this end, we replicated this experiment using tetanus toxoid vaccine, live attenuated measles vaccine, and subunit influenza vaccine.

For the tetanus toxoid study, tetanus toxoid vaccine was administered ID to Balb/c mice either as a bolus (Bolus-P-ID) or via repeated ID injections over 28 days (ED-28day-

ID). Anti-tetanus toxoid IgG titers at day 28 were induced using extended delivery of the vaccine compared to bolus delivery (Figure 5.9b, $p<0.0001$). This shows that extended delivery can improve immune responses to a toxoid vaccine.

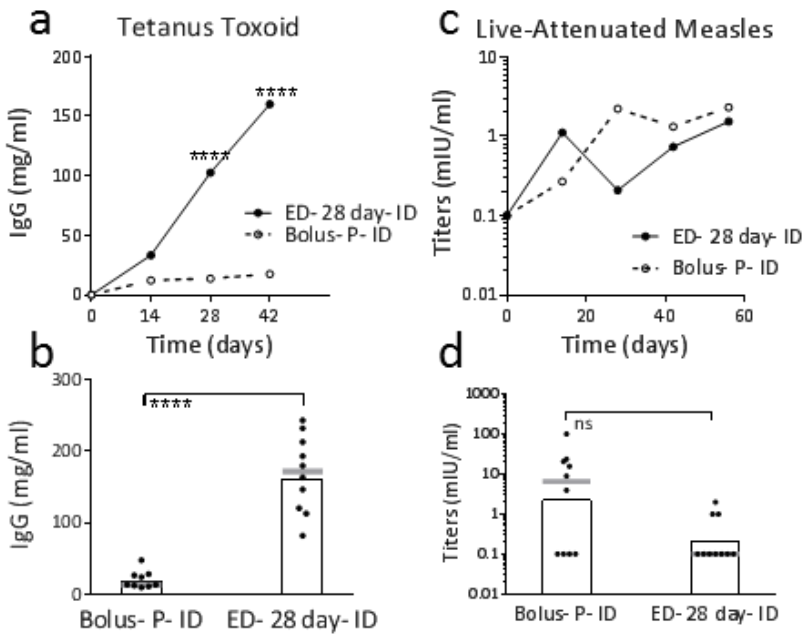

Figure 5.9. Applicability to multiple diseases.
(a) IgG titers following extended delivery of tetanus toxoid into the skin are significantly higher than titers following a bolus delivery (** $p<0.0001$). (b) Day 42 IgG titers following extended delivery of tetanus toxoid into the skin are significantly higher than titers following a bolus delivery (**** $p<0.0001$). (c) Measles virus neutralizing antibody titers over time were determined using a plaque reduction neutralization assay. (d) Titers at day 28 were not significantly different between the two groups (ns $p>0.05$). Total dose for tetanus vaccine was 100% of a human dose (5Lf); total dose for measles vaccine was 20% of a human dose ($200TCID_{50}$).**

Live-attenuated measles vaccine was administered intradermally to cotton rats. The bolus and extended delivery over 28 days were indistinguishable from each other (Figure 5.9d, $p=0.700$), indicating that extended delivery did not improve immunogenicity of this live-attenuated vaccine. This may be because the live-attenuated measles vaccine already effectively mimics an infection with extended presentation of antigen.

In the influenza vaccine study, subunit vaccine was delivered by intradermal injection. Bolus groups were boosted on day 77; hemagglutinin inhibition (HAI) titers were measured at days 28 and 107. Before the boost, HAI titers for the extended delivery group (ED-28 day-ID) was significantly higher than the bolus prime + boost group (Bolus-P+B-ID) (Figure 5.10, p=0.006). After the bolus group received the boost, the higher HAI titers were comparable to the extended delivery group (p=0.695). These data indicate that extended delivery can improve responses to a sub-unit vaccine and reduce the number of doses necessary to achieve an immune response above levels that are protective in humans [196].

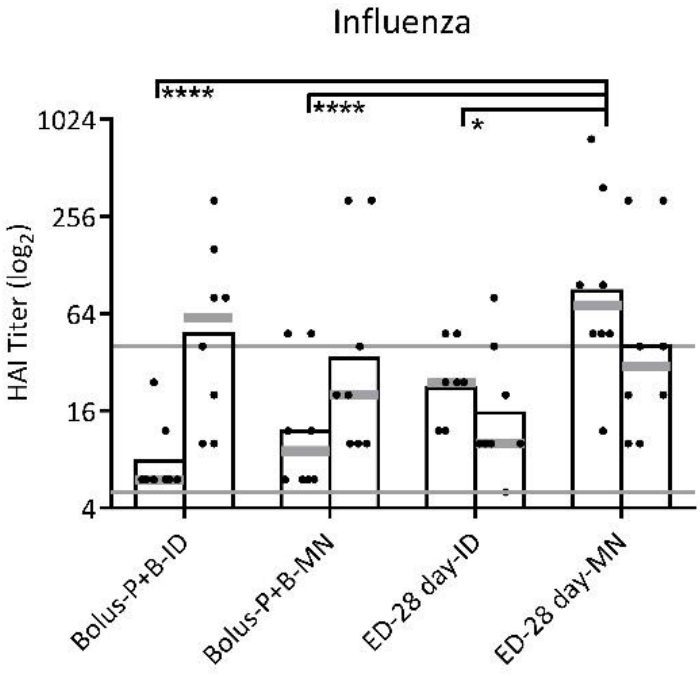

Figure 5.10. Subunit influenza vaccine- Titers at day 56 and 107.
HAI titers pre-boost on day 56 and post-boost on day 107 show higher titers for a single dose of the extended delivery compared to a single bolus dose (*p<0.05, ***p<0.001). Following the boost, HAI titers were equivalent across all groups. The solid line at 5 indicates the detection limit, and the line at 40 represents titer which is considered seropositive. Total dose was 6.7% of a human dose (1ug).

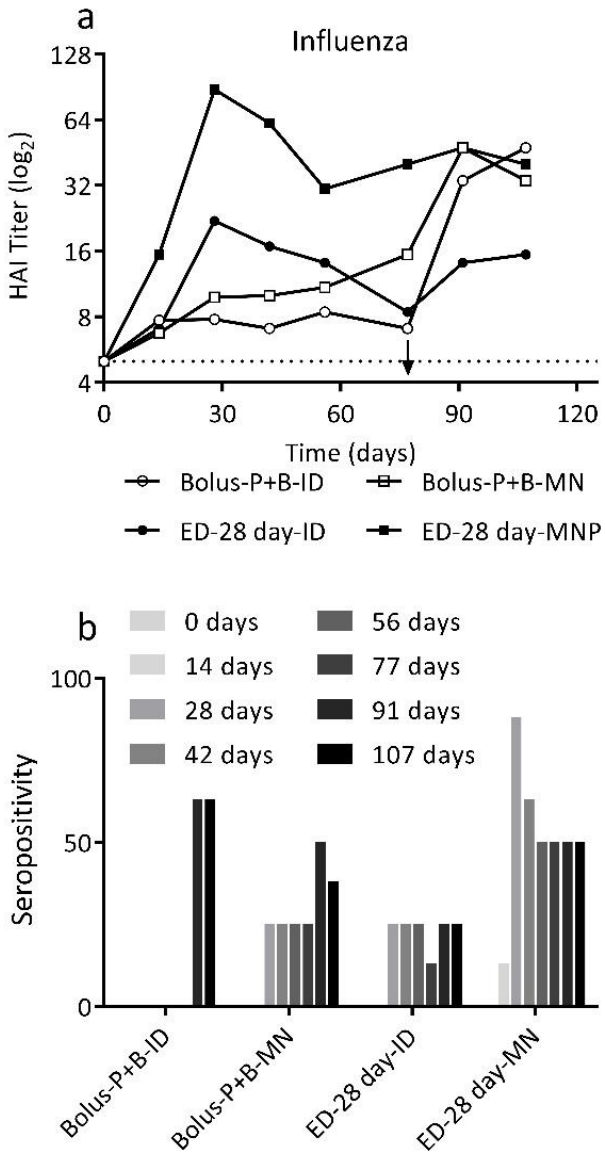

Figure 5.11. Subunit influenza vaccine- Titers over time and seropositivity.
(a) HAI titers over time, with the arrow representing the boost on day 77 and the dotted line representing the baseline of the assay. (b) Seropositivity (titers>40) for each group is shown for each time point.

5.3.5 Delivery using microneedle patches

MN patches have been shown to effectively deliver vaccines into skin and to increase immune responses compared to injections. Here, we administered MN patches loaded with subunit influenza vaccine to Balb/c mice either as a bolus or extended delivery

over 28 days. Bolus groups vaccinated via MN patch or ID injection were boosted on Day 77. Before the boost, extended delivery using MN patches induced higher HAI titers compared to bolus vaccination by intradermal injection or MN patch (Figure 5.10, p=0.025 and 0.037, respectively). Furthermore, mice vaccinated via MN patch extended delivery had higher HAI titers at day 28 compared to repeated ID injections (Figure 5.11). After the boost, the HAI titers for mice boosted with a second bolus dose are not significantly different compared to the single dose extended delivery groups (p>0.6). This shows that extended delivery of a single dose of influenza vaccine given by MN patch can produce immunity similar to two bolus doses of vaccine.

5.3.6 Extended delivery induces a balanced immune response

Motivated by increases in antibody titers, we performed a series of experiments to further understand the effects of long-term antigen presentation on the immune response. In this study, we compared extended delivery using MN patches (ED-28day-MN) to a bolus MN patch (Bolus-P-MN), bolus ID injection (Bolus-P-ID), and a prime + boost group (Bolus-P+B-ID). Additional controls were added: one group received a bolus vaccination via MN patch on day 0 followed by placebo patches for the remainder of the 28 days (Bolus-P-MN + ED-Placebo-MN); another group just received a placebo patch on day 0 (Bolus-Placebo-MN). The former group is meant to look at the effect of repeated injury after skin vaccination (which is an inherent part of the extended delivery method used in this study). Serum antibody titers were measured weekly, while the cellular responses were measured at day 35, which was one week after the final vaccination for the ED group and one week after the boost.

First, we analyzed the IgG, IgG1, and IgG2a of mice vaccinated with subunit influenza vaccine. In all cases titers increased with time. Total influenza IgG titers were highest in mice that received extended delivery with MN patches compared to all other groups, including the bolus prime + boost group (Figure 5.12a, $p<0.05$). These mice also had the highest IgG1 and IgG2a titers (Figure 5.12c and d, $p<0.0001$). The ratio of IgG1/IgG2a was similar among all of the MN patch groups, and were about 10-fold higher than the ID vaccination groups (Figure 5.12b).

All animals were euthanized on day 35, and bone marrow, lymph nodes, and spleen were collected and analyzed for cellular responses. Strong germinal center (GC) and T follicular helper cell (Tfh) responses are desirable for high affinity antibody and long-lived antibody secreting plasma cell (ASC) responses. Using flow cytometry, we examined the GC B cells (GC B) and Tfh in the lymph nodes at day 35. Extended delivery with MN patches generated the strongest responses in these cell populations, which was significantly higher than the ID and MN patch bolus groups (Figure 5.12e and f, $p=0.008$ and 0.003, respectively).

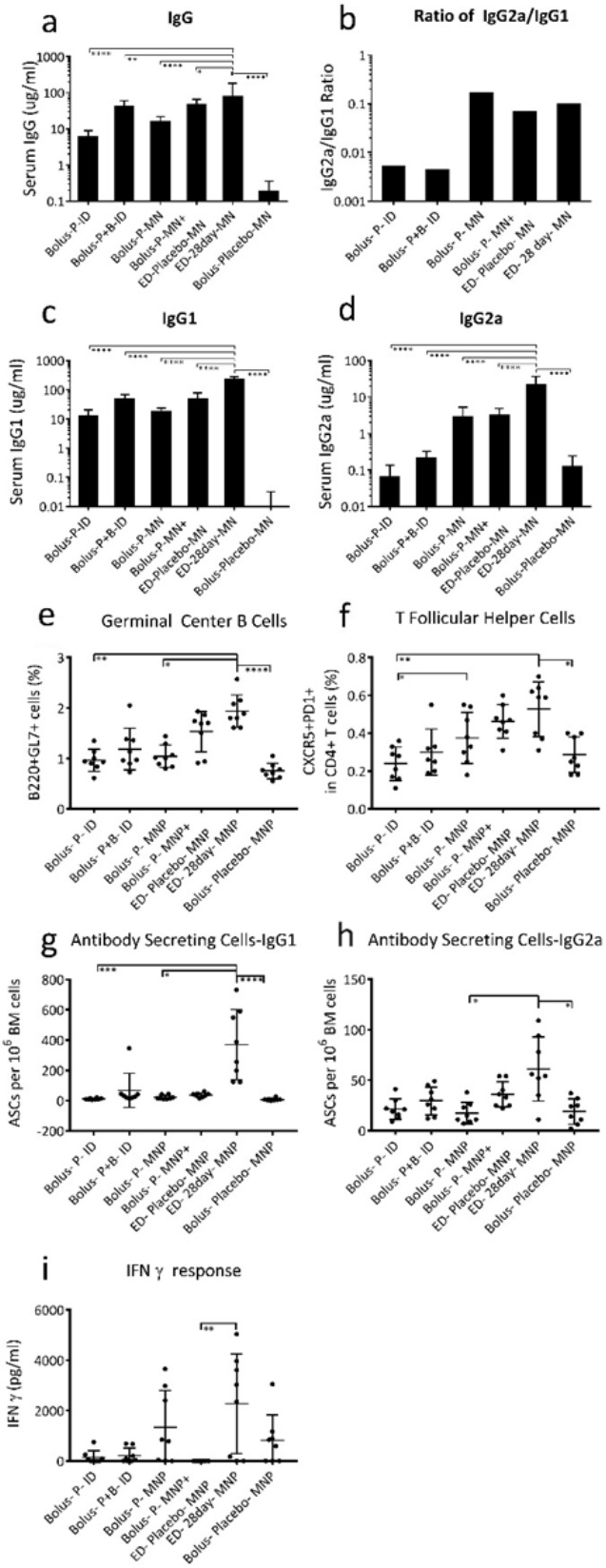

Figure 5.12. Humoral and cellular response.

Serum IgG (a), IgG1 (c), and IgG2a (d) are shown at day 35. Extended delivery with MN patches induced higher antibody titers compared to all other groups. The ratio of IgG1 to IgG2a (b) were approximately equivalent for all MN groups. Lymph nodes had higher percentage of germinal center B cells (e) and T follicular helper cells (f) compared to mice vaccinated by intradermal injection, but not higher than those receiving a MN prime dose followed by placebo patches. Antibody secreting cells for IgG1 (g) and IgG2a (h) were present in higher numbers in the bone marrow in mice vaccinated with extended delivery (MN-ED) than bolus MN patch vaccination. (i) Splenocytes that were restimulated with antigen secreted higher concentrations of IFN ɣ after extended delivery vaccination. Plasmablasts in the lymph nodes were comparable at day 35 (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). Total dose was 6.7% of a human dose (1ug).

ASCs go to the bone marrow following a vaccination or infection and are associated with long-term memory response and antibody production [197]. Both IgG1 and IgG2a ASCs in the bone marrow were significantly more prevalent after extended delivery by MN patch than bolus vaccination by MN patch (Figure 5.12g and h, $p=0.024$ and 0.010, respectively). While ASCs, GC B, and Tfh responses were stronger after extended delivery by MN patch compared to the bolus MN patch followed by repeated placebo patches, this difference was not statistically different in this small data set (Figure 5.12e-h, $p>0.999$). This suggests that repeated injury from MN patch application may play a role in the immune response seen after extended delivery vaccination. Plasmablast levels in the lymph nodes on day 35 were not significantly different across all groups after vaccination (Figure 5.13). This lack of differences in responses may be because measurement at day 35 was too late in the immune response process as suggested by prior literature [109].

Finally, splenocytes were re-stimulated with antigen, and the supernatant was analyzed for IFN-gamma level by ELISA. Again, extended delivery by MN patch showed the strongest IFN ɣ response, indicating a strong Th1 cellular response. IFN ɣ levels were elevated following extended delivery with MN patches compared to all other groups, although this difference was not significant in most cases (Figure 5.12i). Taken together,

extended delivery vaccination with MN patches amplified the humoral immune response through increased antigen-specific antibody, GC B cell, Tfh, ASC and Th1 responses.

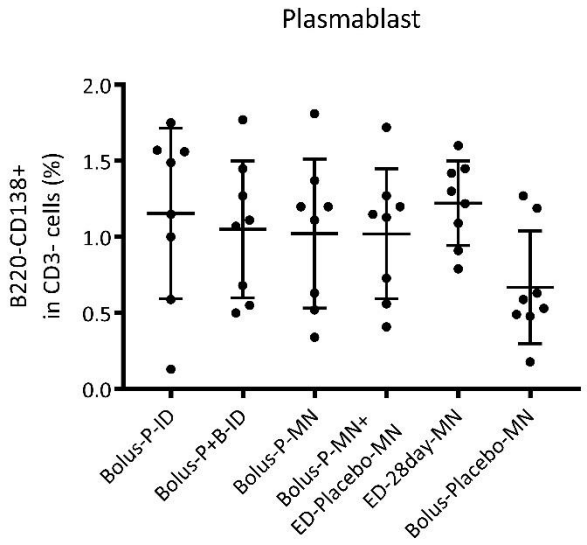

Figure 5.13. Plasmablast response.
Plasmablasts in the lymph nodes were comparable at day 35.

5.4 Discussion

While vaccines provide one of the most powerful and cost-effective tools in public health, insufficient vaccination coverage limits the impact of vaccines. Studies show that 1.5 million lives could be saved by improved vaccination coverage, both in terms of first-dose vaccination and completing vaccination series [172]. One way to improve vaccination coverage is to reduce the number of vaccine doses so that, for example, a single vaccination can provide full protection without the need for booster doses. Vaccines could also improve impact on public health by increasing vaccine immunogenicity without the added cost of increasing vaccine dose or the use of adjuvants. For those vaccine that already generate robust immune response in current formulations, increased immunogenicity could enable cost savings by lowering the dose administered. Finally, the reach of vaccines could be

improved by simplifying the logistics of vaccination, such as less reliance on cold-chain refrigeration, avoidance of biohazardous sharps waste and reduced need for highly trained healthcare personnel to administer vaccines.

With these goals in mind, this study sought identify ways to increase vaccine immunogenicity without increasing vaccine dose, reduce the number of doses needed to achieve protective immunity and assess the possible use of simplified vaccination methods. We did so by studying the effect of kinetic profile of vaccine release into the skin on immune response during extended delivery, assessing the potential to increase immunogenicity and/or reduce the number of doses using four licensed vaccines of significance to public health, and to further understand the immune response to extended vaccine delivery.

5.4.1 Vaccine delivery profile

The concept of extended release of vaccines has been utilized previously to have an adjuvant effect; however, in most cases, the systems were developed to prolong exposure and follow similar release profiles with an initial burst followed by low levels of release [39, 89, 93]. Few have studied how differences in antigen profiles can affect the immune response. We began by using inactivated polio vaccine (IPV) to look at the optimal profile and length of release. From Figure 5.2, Constant and Exponentially Increasing profiles induced higher neutralizing antibody titers compared to single bolus injection. These results indicate that vaccination profiles that roughly follow natural infection kinetics are preferred over bolus or profiles with an initial burst. Other studies using model protein antigens found that using natural infection kinetics can improve the immune

responses over bolus injections [92, 109, 113]. Some studies have suggested that these kinetic profiles induce better T-cell stimulation and prolongs T-cell memory for a particular antigen, indicating activation of both cellular and humoral arms of the immune system [89].

We also identified that longer stimulations of the immune response can elicit better responses, with release over one month performing better than release over one or two weeks. It is possible that even longer exposures could induce stronger reactions, although an extremely low vaccine release rate over a longer time could be insufficient to generate an immune response or could even induce tolerance to the antigen [198, 199].

Today, most vaccines are administered IM; previous work has demonstrated that controlled-release systems such as microparticles given via IM injection produce comparable results to subcutaneous and intranasal delivery [200] [201]. Vaccine delivery into the skin is becoming a more promising option due to the potential for enhanced immune response for some vaccines in the skin and the ease of delivery into the skin with novel devices [158, 202, 203]; however, no studies have directly compared IM and ID vaccination with extended delivery. Here, we showed that administering daily doses into the skin or into the muscle induced comparable immune responses, both of which were superior to bolus doses. This result motivates the development of a controlled release MN patch that could ease the logistical challenges of vaccinations by reducing the total number of doses and by simplifying the vaccination administration, as discussed below.

Compared to inactivated vaccines, live-attenuated vaccines are known to induce potent immune responses and require fewer boosters to provide long-lasting protection. As the vaccine infects and replicates in its host, the host's exposure to the vaccine is prolonged,

innately providing extended exposure to the antigen for roughly 14 days [204]. Therefore, we predicted that prolonging antigen delivery through repeated injections will not be advantageous for a live-attenuated vaccine. Consistent with this hypothesis, the live-attenuated measles vaccine did not induce a more potent immune response with extended delivery when compared to bolus vaccination, whereas the three inactivated or protein-based vaccines did.

5.4.2 Improved immunogenicity of licensed vaccines

In this study, extended delivery improved immunogenicity of three licensed vaccines: IPV, tetanus toxoid and subunit influenza vaccines. This suggests that our finding may be applicable to a broad range of non-replicating vaccines of significance to public health.

5.4.2.1 Inactivated polio vaccine

As the polio eradication initiative approaches its conclusion, IPV is being increasingly administered across the world. Vaccine shortages as well as the relatively high cost of IPV has led to a WHO recommendation for use of a one-fifth dose of IPV administered ID as a two-dose regimen that than the conventional three full-strength doses administered IM [205]. The improved immunogenicity of IPV when administered ID by extended delivery could enable further dose reduction and/or reduce the number of vaccine doses, possibly reducing the two-dose ID regimen to a single dose.

5.4.2.2 Tetanus toxoid

Tetanus toxoid is currently given as a multi-dose regimen with 5 – 6 doses in childhood followed, in some cases, by repeated boosters in adulthood and during pregnancy [122]. Extended delivery of tetanus toxoid could reduce the number of doses and/or frequency of boosters, thereby simplifying the vaccination schedule that includes vaccination older children and adults outside the primary childhood vaccination schedule.

5.4.2.3 Influenza vaccine

Influenza vaccine is given as a two-dose regimen upon first vaccination of children, followed by annual re-vaccination for life where high-dose vaccination is recommended in the elderly [206]. Extended-delivery vaccination could eliminate the need for two doses upon first vaccination, reduce the doses needed during annual vaccination campaigns including the high-dose vaccine administered to the elderly.

5.4.3 Microneedle patches allow for extended delivery

In this study, we were motivated to study extended delivery in the skin because of the increased immunogenicity of skin vaccination[27, 41, 43, 45, 167], as well as the opportunity to use MN patches that not only target skin, but can also simplify vaccination logistics [26, 158]. While administering intradermal injections via the Mantoux method requires an experienced provider, MN patches are a novel method to easily vaccinate large numbers of people. Vaccination via MN patches has been previously shown to induce a potent immune response [5, 9, 13, 28, 45, 161]. Here for the first time, we demonstrated that daily vaccination with dissolving MN patches produced even higher HAI titers compared to daily intradermal injections. We hypothesize that this is due to the differences in delivered volume and viscosities. Injections are a large volume of saline forced into the

skin at a high pressure. The saline and vaccine quickly disperse throughout the skin, and the vaccine can be cleared by immune cells. Conversely, an inserted microneedle patch contains sugars and polymers which can form a transient gel in the skin [115, 207]. The polymers used here have been shown to dissolve and release their cargo on the order of hours. This work motivates future development of a single microneedle patch which can slowly dispense vaccine over the course of one month.

5.4.4 Immune response to extended delivery

In order to characterize the immune response, we analyzed the IgG, IgG1, and IgG2a isotypes in the mouse serum after influenza vaccination. We note that vaccine administered over the course of one month induced higher titers across all isotypes [107, 208-210]. Studies using controlled release of vaccines have found increases in both IgG1 and IgG2a titers; however, there is no clear consensus on whether the response is skewed towards a Th1 [211, 212] or Th2 response [209, 213, 214]. Strong GC reaction and Tfh cell response are known to generate high affinity antibody, isotype switching and lasting immune memory [215]. Repeated administration of MN patches had increased levels of GC B and Tfh cells in lymph nodes and antibody secreting plasma cells in bone marrow, suggesting strong humoral and memory responses. Also, repeated MN patches had high Th1 response. High GC B and Tfh cell numbers along with high serum antibody titers have been seen in previous MN patch work and have shown to be correlated with rapid virus clearance and survival following lethal challenge [45]. Daily injected vaccination and slow release formulations with MN patches have demonstrated increased CD8+ T-cells

responses [92, 115, 117]. Taken together, repeated application of MN patches elicits superior humoral and cellular immunity compared to bolus vaccinations.

We additionally noted increased responses in the bolus MN patch vaccination followed by repeated placebo MN patches. While this response was not as great as repeated vaccination, it was superior to bolus MN patch vaccination. This suggests that repeated injury to the site of vaccination is enhancing the immune response and that the increase in the extended delivery group is not solely due to antigen exposure. Repeated application of MN patches did not induce increased infection or inflammation markers [118]; however, prolonged presence of antigen following MN patch delivery increased local inflammation [117]. Additionally, there is evidence that colocalization of the antigen with cell death can enhance the humoral response and CD8+ T-cell induction [216, 217]. More work will need to be done to determine the role of repeated injury at the site of vaccination on the immune response.

In this work, we demonstrated the impact of extended delivery of vaccines by utilizing daily intradermal injections. These injections allowed us to replicate different release profiles without the added complications of utilizing different controlled release systems. While repeated injections are not a practical method to administer vaccines, this work motivates the development of controlled release systems for a single vaccination. Injectable controlled release systems such as poly(lactic-co-glycolic acid) (PLGA) microparticles and thermosensitve hydrogels have been shown to be able to release proteins over the course of weeks to months [218, 219]. However, an even stronger

approach would be to combine the benefits of a controlled release system with those of microneedle patches [33]. Using controlled release formulations in microneedle patches has been previously accomplished using PLGA, poly(acrylic acid), and silk, among others, although these patches elicit significant burst releases that we have shown to be a detriment to inducing a large immune response [115-117]. Novel MN patch formulations are necessary to achieve single vaccination without this burst.

5.5 Conclusion

In conclusion, this study showed that extended delivery of vaccines to the skin enabled a single dose of vaccine delivered over the course of one month to provide similar immune responses to vaccination using two bolus doses in a prime plus boost protocol. This method improved the immune response to three different killed vaccines, but not to a live vaccine. Furthermore, we demonstrated that the humoral response lasted at least six months and that delivering the doses via dissolving MN patches increased both the humoral and cellular responses to the vaccines. Antigen extended delivery has the potential for dose sparing or eliminating the number of boosts required to reach protective levels.

CHAPTER 6. DISCUSSION

Vaccinations have been one of the most important life-saving health care technologies in the last century. Immunizations prevent between two and three million deaths each year [3]; however, an addition 1.5 million lives could be saved each year with increased vaccination coverage. Apart from oral polio, rotavirus, and cholera vaccines and intradermal Bacille Calmette-Guérin vaccine, all currently recommended vaccines by the WHO require administration with a needle and syringe to deliver the vaccine into the muscle or subcutaneous space. This technology creates numerous logistical problems that can be costly during vaccination campaigns. Briefly, the needle and syringes must be properly disposed after vaccination; trained healthcare providers are required to reconstitute and administer the vaccines; the cold chain, an expensive series of refrigerators, is required to maintain viral activity during shipping and storage; and multiple doses of vaccines may be necessary to invoke a long-lasting immunity to an antigen.

In this work, we developed microneedle (MN) patches to replace the needle and syringe when administering vaccines. Dissolving MN patches are single dose, single use patches that painlessly deliver the vaccines into the skin. Once dissolved, the needles can no longer be used, and the remaining backing can be discarded as regular waste. Patches can be administered by minimally trained personnel or even self-administered. These patches can be stored outside the cold chain for extended periods of time. With some vaccines, the intradermal vaccination improves the immune response allowing for dose sparing. However, these patches must be formulated and tested for each vaccine antigen delivered.

This work sought to fill two gaps of knowledge in the MN space. The first was the development of a MN patch to deliver measles and rubella (MR) vaccines. These live-attenuated vaccines are among the most unstable vaccines that are currently licensed [25]. Additionally, two doses of the vaccine are necessary to achieve high (>95%) population immunity, and the first dose cannot be administered until 12 months of life [47]. In the second part of this work, we utilized extended presentation of an antigen as a method to improve the immune response to vaccination with an injection or with a MN patch. Many vaccines such as inactivated polio vaccine or tetanus vaccine require two or more doses to be effective; while other vaccines such as seasonal influenza vaccine suffer from low efficacy, limiting the protectiveness of vaccination.

In the first chapter, we developed thermostable MR MN patches. Buffers were crucial to maintain rubella vaccine activity during drying, but were not essential for measles vaccine. Various sugars, amino acids, and proteins can be used to stabilize MR. A combination of sucrose and threonine were used to demonstrate that MR MN patches could withstand long term storage at elevated temperatures. The thermostability allows the patches to be stored outside the cold chain. The necessity to maintain 2-8˚C during shipment and storage accounts for a significant portion of the cost of single vaccination and leads to substantial vaccine wastage. Utilizing the formulations developed here, patches can be shipped without refrigeration, reducing the cost of vaccinations. This will also allow for vaccination in more remote areas that currently have trouble accessing vaccinations.

Juvenile and infant rhesus macaques were vaccinated using MR MN patches to assess the safety and immunogenicity of the patches as compared to a subcutaneous

vaccination. After MN patch administration, slight redness at the site was present for about an hour, but no adverse events were noted. The humoral response was studied after vaccination and after measles viral challenge; all data demonstrated that the MN patches induced potent immune responses to both vaccines that was equivalent to the subcutaneous vaccination. Unfortunately, MN patches were unable to overcome the presence of maternal antibodies and induced an inferior response compared to naïve vaccinations. Overall, MR MN patches were safe and immunogenic in a rhesus macaque model. This was the first time that infant rhesus macaques had been vaccinated with MN patches. Young immune systems are still developing and have weaker responses to vaccination. However, this work demonstrated the formation of a robust, protective response that was present at least six months after vaccination. This indicates that MN patches could be used in high-risk populations such as young, old, and immune suppressed. Taken together, these data support that vaccination utilizing a MN patch to deliver MR vaccines and that these patches could be beneficial in eradication efforts.

In the second set of experiments, we studied the immune response to extended delivery of a vaccine antigen. We were inspired by different types of vaccines. Live-attenuated vaccines, such as MR, must replicate in the patient and provide life-long protection against the pathogen. Inactivated or subunit vaccines, on the other hand, are safer but do not always induce strong responses. Inactivated polio vaccine (IPV) must be administered at least 3 times for 95% of the population to respond [220]. Through this work, we sought to combine the safety of inactivated vaccines with the immune response of a replicating vaccine. We hypothesized that kinetics that mimicked natural infections would induce a more potent immune response. Using daily intradermal injections or MN

patches, a zeroth order, constant release profile over 28 days induced the most potent immune response. The improved immune response is seen over a range of inactivated vaccine types: IPV, tetanus toxoid, and subunit influenza vaccine; however, extended delivery of live-attenuated measles vaccine did not enhance the immune response compared to a bolus injection. Furthermore, daily MN patches delivering flu antigen induced strong neutralizing antibodies and cellular response. Future work could address formulation of MN patches for extended vaccine delivery after application of a single patch.

By implementing antigen extended delivery in vaccination strategies, the improved immune response to a single dose could increase the number of people protected against various pathogens. This technique can lower the required number of doses for seroconversion. For vaccination campaigns and elimination/eradication efforts, reaching populations in rural or politically unstable areas is particularly difficult. Limiting the number of times vaccinators must travel to these areas can save money and allow for better allocation of resources to achieve elimination/eradication goals. Extended delivery could also allow for dose sparing, or using a lower dose to achieve an equivalent immune response. Dose sparing can save antigen during vaccine shortages, such as manufacturing malfunction or pandemic flu outbreak, while maximizing the number of people vaccinated. Thus, extended delivery can improve vaccination coverage and reduce vaccine-preventable deaths worldwide.

In this work, dissolving MN patches were developed and tested for thermostability and strong immunogenicity. There remains a need to develop the manufacturing technology to increase production for dissolving MN patches. Other technologies are under

development for intradermal delivery of vaccines. Coated MN patches, consisting of a vaccine solution dried onto metal MNs, are comparable to dissolving MN patches in many aspects; however, the needles remain sharp after insertion, which could lead to disease spread through needle re-use and improper disposal [221]. Particle and jet injectors utilize a high-pressured stream of gas or liquid to push vaccines through the upper layers of the skin. These benefit vaccination campaigns by removing any sharps waste; jet injectors can utilize current formulations while particle injectors would require reformulation. External pressure-generating devices and significant nurse training are required to administer these vaccines. Jet injectors have been used in clinical trials to mixed success [185, 222]. Other methods for delivering vaccines, such as electroporation, ultrasound, and thermal/chemical abrasion, are underdevelopment but may be cost prohibitive for developing countries [185].

Vaccinations hold great potential in reducing the global disease burden. However, current technologies hamper delivering the life-saving vaccines. Novel vaccine delivery technologies will be a key part in reaching high vaccination coverage and eradication of polio, measles, rubella, and other viruses. Dissolving MN patches can reduce the cost and ease logistics for vaccinations, especially in developing countries. These patches eliminate biohazardous sharps waste, are well tolerated by patients, and can be administered by minimally trained personnel. These patches can be removed from the cold chain and induce potent, long-lasting responses. Thus, MN patches have great potential to safely and effectively deliver vaccines and increase vaccination coverage.

CHAPTER 7. FUTURE DIRECTIONS

7.1 Add mumps vaccine to the measles-rubella microneedle patch

In this project, thermostable, immunogenic measles-rubella (MR) microneedle (MN) patches were developed. Currently, these vaccines are co-administered with live-attenuated mumps vaccine (measles-mumps-rubella (MMR)). The World Health Organization (WHO) has stated that mumps could be eliminated if two-dose vaccine coverage were increased; however, mumps has not received as many resources compared to MR based on global mortality and disease burden [223-225]. Routine mumps vaccination is recommended in countries with established MR vaccination coverage (>80%), and two doses are necessary for high levels of immunity in a community [225].

Mumps vaccine has been delivered as part of an MMR formulation by jet injector [58, 60, 226] and aerosol [66]. Clinical trials have demonstrated positive immune response data for these alternative delivery methods, suggesting that delivery of mumps vaccine with a MN patch would induce a potent immune response. Adding novel vaccines to a MN patch requires reformulation and evaluation of said patch to ensure that activity of all vaccine components is retained. Compared to measles vaccine, mumps vaccine has been shown to be more resilient to centrifugation for filtration, low pH, and elevated temperature in liquid state [77]. However, little data exists of the stability of mumps vaccine during drying and storage. Because the current licensed product is a lyophilized vial, we can predict that the vaccine can withstand MN patch manufacturing. More studies are necessary to predict the potential for long-term storage in the MN patch.

7.2 Improve the stability of the measles-rubella vaccine microneedle patch

Maintaining vaccine activity during storage is critical to the success of MN patches in the developing world. The current lyophilized vials are have a shelf life of 2 years at 2-8˚C or one month at 37˚C, in which vaccine activity decreases by 1 log [55]. In our experiments, MR MN patches were stable at 40˚C for up to one month with no loss and up to two months with 1 log loss of activity. Improving the stability at elevated temperatures would allow the patches to travel further, thereby improving access to more rural areas [49]. Screening studies are time and resource intensive; carefully selecting the parameters to test is crucial to ensuring rapid success. We only worked with combinations that were equal mass ratios, but other work in the group has demonstrated that the ratio of the excipients can play a critical role in the stability of the vaccine (inactivated polio vaccine, unpublished). Additionally, in other studies, divalent cations were proven helpful in stabilization during spray drying measles vaccine [74]. Including these cations with sugar/amino acid combinations may improve stability during manufacturing and storage. Any novel formulations should be tested for their stabilizing nature during short and long-term storage. Additionally, MN patches could be examined for their mechanical strength after storage. Previous work has demonstrated that MNs remained sharp after storage for one year with desiccant [9]; however, this may change based on patch formulation. Finally, only *in vitro* cell infectivity assays have been used to assess stability. An *in vivo* study in cotton rats or rhesus macaques could be used to further demonstrate that patches retained viral activity and immunogenicity after extended storage.

7.3 Prepare the measles-rubella microneedle patch for clinical trials

At the conclusion of this project, efforts are underway to scale up the MR MN patches for a phase 1 clinical trial. To date, influenza vaccine is the only vaccine to be administered via a dissolving MN patch in a clinical trial. These studies demonstrated that the MN patch was safe and well-tolerated by the patients and were able to induce a robust immune response [6, 16].

To meet the demand for a clinical trial and eventual market release, the manufacturing process used in this work will need to be adjusted. The methods used in this work are labor intensive, low throughput, and can lead to vaccine wastage and variable dose loading. Novel techniques could be used to allow more precise MN manufacturing. Filling PDMS molds using a roller method [227] could reduce the volume required for patch manufacturing, as well as ensure the vaccine is near the tips for higher delivery efficiencies. Dissolving MN patches have been 3-D printed, offering flexibility in MN shape, dose loading, and dose distribution within the MN [228]. However, no one has yet loaded vaccines into these patches; it is unknown if the vaccine would be able to withstand this manufacturing process. In addition, quality assurance and control mechanisms must be put in place to ensure proper sterilization of the MN patches and consistent dosing [15, 229].

7.4 Fabricate controlled release patch for single administration

In this work, we demonstrated that daily microneedle patches improve the immune response compared to a single vaccination. These patches could be provided in a pre-packaged set, and the patient could apply the patches daily [16]. However, this strategy is not practical, especially in a low resource setting. A novel controlled release MN patch

requiring only a single administration could increase patient compliance. The MN patch would need to quickly dissolve from the backing, releasing the MNs into the skin. Then, the MNs could slowly release vaccine over the course of one month.

Numerous MN systems have been designed to slowly release proteins at various rates. These systems can be divided into two categories: (1) the needles break off to act as a reservoir in the skin or (2) particles are loaded into the needles, surrounded by faster dissolving matrix. In the first scenario, full needles were cast of the drug and a polymer such as poly(lactic-co-glycolic acid) (PLGA), and a metal or rapidly dissolving polymeric support structure was used to insert the needles fully into the skin [115, 116, 140]. Other similar systems have been developed to deliver entire needles into the skin [230]. Another method is to encapsulate micro- or nanoparticles into the MN patches. Various water-soluble polymers and sugars can entrap the particles in the MN array and provide the mechanical strength necessary to pierce the skin. Once the polymers and sugars dissolve, the particles remain in the skin and can release their cargo at a determined rate [7, 115]. These particles could be coated onto metal structures using increased viscosity [231] or layer-by-layer assembly [232].

One unknown of this work is how the reservoir will remain in the skin during the release. Skin cells are constantly being replaced in the basal layer of the epidermis and are then pushed into the upper layers as the older cells die and are removed. This process can take approximately 20-40 days [233, 234]. To date, particles have delivered antigen in the skin continuously for up to two weeks [115, 235].

CHAPTER 8. CONCLUSION

MN patches are an innovative technology to administer vaccines without needles and syringes. With its numerous advantages, these patches can reduce costs and ease logistics for vaccinations. When used in eradication efforts, the patches developed here can reduce the necessary resources for vaccination campaigns, freeing those resources for more campaigns or other activities such as surveillance and outbreak response. In this work, we developed the first measles-rubella (MR) vaccine MN patch. This patch was thermostable, raised no significant safety concerns, and induced potent immune responses in infant and juvenile rhesus macaques. In the second part of this thesis, daily exposure to small doses of a vaccine induced higher neutralizing antibodies compared to a bolus vaccination. This work enhances the MN community's understanding of vaccine stability and intradermal vaccination and can serve as a powerful tool to expand global access to vaccinations.

APPENDIX A. MEASLES- RUBELLA STABILITY TABLES

Table 1. Single excipients stored from one week at elevated temperature. Vaccines were dried with at room temperature overnight and stored with desiccant for one week at 40°C (Figure 3.3). The activity after drying is reported as the percent activity compared to the liquid control. The activity loss during storage is reported as the percent activity at day 7 compared to the activity at day 0. The excipients that were used in the next phase are highlighted.

Excipient	Measles		Rubella	
	% at Day 0 to Liquid Control	% at Day 7 to Day 0	% at Day 0 to Liquid Control	% at Day 7 to Day 0
Na Lactate	19.1%	0.0%	28.0%	1.5%
Histidine	1.0%	35.5%	32.9%	9.0%
Dextrose	23.7%	0.0%	32.9%	0.0%
Asparigine	3.8%	0.0%	13.9%	7.6%
Threonine	1.6%	0.0%	55.9%	1.5%
Glycine	0.5%	0.0%	35.2%	1.2%
Adonitol	26.3%	0.0%	25.7%	0.0%
Serine	11.7%	0.0%	20.2%	2.1%
Na Citrate	0.8%	0.0%	4.6%	0.0%
Na Thiosulfate	27.9%	0.0%	4.8%	0.0%
Sucrose	8.4%	6.9%	29.4%	22.7%
K Gluconate	4.0%	0.0%	116.8%	4.7%
Sorbitol	9.9%	0.0%	144.3%	4.2%
Na Gluconate	15.2%	0.0%	154.1%	3.6%
Maltose	0.7%	0.0%	87.6%	0.7%
Glucose	3.2%	0.0%	53.7%	0.0%
Mannitol	0.3%	0.0%	50.6%	4.2%
K Citrate	0.0%	0.0%	55.1%	0.0%
Myo-inositol	1.2%	0.0%	35.7%	5.0%
MADPG	0.3%	0.0%	12.0%	0.0%
Trehalose	3.7%	20.8%	110.3%	8.5%
Na Phosphate	0.5%	0.0%	0.0%	0.0%

Table 2. Measles vaccine combination screen.
Measles vaccine was dried in dual excipient combinations overnight at room temperature and stored for one week at 40°C. The activity remaining at day 0 and 7 are shown in Figure 3.4(top).

Excipient 1	Excipient 2	Code	% Day 0 to liquid control	t-test liquid control and Day 0	Is p>0.05?	% Day 7 to Day 0	t-test Day 0 and Day 7	Is p>0.05?
Histidine	Sucrose	H-S	26 %	0.071	Y	29%	0.126	Y
Histidine	Trehalose	H-T	12%	0.036	N	27%	0.374	Y
Histidine	Asparagine	H-A	3%	0.023	N	8%	0.379	Y
Histidine	Maltose	H-M	13%	0.039	N	0%	0.146	Y
Histidine	Sorbitol	H-So	15%	0.041	N	4%	0.076	Y
Histidine	K Gluconate	K-KG	13%	0.039	N	0%	0.146	Y
Histidine	Dextrose	H-D	7%	0.028	N	0%	0.013	N
Histidine	Na Gluconate	H-NaG	26%	0.071	Y	6%	0.249	Y
Sucrose	Trehalose	S-T	7%	0.028	N	11%	0.019	N
Sucrose	Asparagine	S-A	33%	0.098	Y	44%	0.045	N
Sucrose	Maltose	S-M	7%	0.029	N	0%	0.034	N
Sucrose	Sorbitol	S-So	9%	0.032	N	0%	0.028	N
Sucrose	K Gluconate	S-KG	13%	0.039	N	3%	0.153	Y
Sucrose	Dextrose	S-D	8%	0.03	N	0%	0.12	Y
Sucrose	Na Gluconate	S-NaG	13%	0.039	N	9%	0.171	Y
Trehalose	Asparagine	T-A	117%	0.7	Y	12%	0.282	Y
Trehalose	Maltose	T-M	7%	0.028	N	0%	0.013	N
Trehalose	Sorbitol	T-So	11%	0.034	N	2%	0	N
Trehalose	K Gluconate	T-KG	8%	0.03	N	0%	0.006	N
Trehalose	Dextrose	T-D	4%	0.025	N	0%	0.194	Y
Trehalose	Na Gluconate	T-NaG	1%	0.022	N	0%	0.028	N
Asparagine	Maltose	A-M	4%	0.025	N	0%	0.194	Y
Asparagine	Sorbitol	A-So	4%	0.025	N	12%	0.037	N
Asparagine	K Gluconate	A-KG	3%	0.024	N	21%	0.337	Y
Asparagine	Dextrose	A-D	3%	0.024	N	0%	0.327	Y
Asparagine	Na Gluconate	A-NaG	5%	0.026	N	13%	0.091	Y
Maltose	Sorbitol	M-So	4%	0.025	N	0%	0.346	Y
Maltose	K Gluconate	M-KG	4%	0.025	N	0%	0.315	Y
Maltose	Dextrose	M-D	1%	0.022	N	0%	0.146	Y
Maltose	Na Gluconate	M-NaG	4%	0.025	N	0%	0.009	N
Sorbitol	K Gluconate	So-KG	5%	0.026	N	0%	0.034	N
Sorbitol	Dextrose	So-D	2%	0.022	N	0%	0.07	Y
Sorbitol	Na Gluconate	So-NaG	4%	0.025	N	0%	0.315	Y
K Gluconate	Dextrose	KG-D	5%	0.026	N	0%	0.07	Y
K Gluconate	Na Gluconate	KG-NaG	22%	0.059	Y	0%	0.194	Y
Dextrose	Na Gluconate	D-NaG	3%	0.024	N	0%	0.256	Y
Sucrose	Threonine	S-Thr	5%	0.027	N	36%	0.3	Y

Table 3. Rubella vaccine combination screen.
Rubella vaccine was dried in a combination of excipients and stored for one week at 40°C. This data is represented graphically in Figure 3.4(bottom).

Excipient 1	Excipient 2	Code	% Day 0 to liquid control	t-test liquid control and Day 0	Is p>0.05?	% Day 7 to Day 0	t-test Day 0 and Day 7	Is p>0.05?
Histidine	Sucrose	H-S	8%	0.08	Y	61%	0.296	Y
Histidine	Trehalose	H-T	33%	0.172	Y	59%	0.08	Y
Histidine	Asparagine	H-A	20%	0.116	Y	147%	0.045	N
Histidine	Maltose	H-M	19%	0.11	Y	135%	0.298	Y
Histidine	Sorbitol	H-So	21%	0.118	Y	53%	0.011	N
Histidine	K Gluconate	K-KG	34%	0.181	Y	24%	0.013	N
Histidine	Dextrose	H-D	19%	0.113	Y	0%	0.024	N
Histidine	Na Gluconate	H-NaG	27%	0.144	Y	30%	0.076	Y
Sucrose	Trehalose	S-T	40%	0.215	Y	234%	0.331	Y
Sucrose	Asparagine	S-A	10%	0.083	Y	157%	0.298	Y
Sucrose	Maltose	S-M	38%	0.206	Y	101%	0.98	Y
Sucrose	Sorbitol	S-So	37%	0.196	Y	192%	0.02	N
Sucrose	K Gluconate	S-KG	24%	0.138	Y	226%	0.333	Y
Sucrose	Dextrose	S-D	31%	0.163	Y	0%	0.056	Y
Sucrose	Na Gluconate	S-NaG	13%	0.093	Y	261%	0.091	Y
Trehalose	Asparagine	T-A	6%	0.074	Y	269%	0.136	Y
Trehalose	Maltose	T-M	36%	0.189	Y	73%	0.192	Y
Trehalose	Sorbitol	T-So	36%	0.192	Y	115%	0.264	Y
Trehalose	K Gluconate	T-KG	57%	0.359	Y	5%	0	N
Trehalose	Dextrose	T-D	39%	0.211	Y	0%	0.025	N
Trehalose	Na Gluconate	T-NaG	44%	0.247	Y	13%	0.016	N
Asparagine	Maltose	A-M	28%	0.149	Y	13%	0.027	N
Asparagine	Sorbitol	A-So	44%	0.246	Y	10%	0.036	N
Asparagine	K Gluconate	A-KG	74%	0.576	Y	9%	0.088	Y
Asparagine	Dextrose	A-D	5%	0.072	Y	0%	0.067	Y
Asparagine	Na Gluconate	A-NaG	7%	0.077	Y	0%	0.015	N
Maltose	Sorbitol	M-So	63%	0.432	Y	0%	0.013	N
Maltose	K Gluconate	M-KG	59%	0.383	Y	7%	0.001	N
Maltose	Dextrose	M-D	52%	0.312	Y	0%	0.036	N
Maltose	Na Gluconate	M-NaG	49%	0.285	Y	6%	0.001	N
Sorbitol	K Gluconate	So-KG	44%	0.244	Y	6%	0.001	N
Sorbitol	Dextrose	So-D	49%	0.284	Y	0%	0.045	N
Sorbitol	Na Gluconate	So-NaG	49%	0.281	Y	19%	0.011	N
K Gluconate	Dextrose	KG-D	33%	0.176	Y	0%	0.015	N
K Gluconate	Na Gluconate	KG-NaG	61%	0.404	Y	13%	0.009	N
Dextrose	Na Gluconate	D-NaG	29%	0.155	Y	0%	0.157	Y
Sucrose	Threonine	S-Thr	51%	0.312	Y	43%	0.249	Y

APPENDIX B. INFANT MACAQUES VACCINATION DATA

Table 4. Experimental design, measles neutralization titers, and measles virus titers in peripheral blood after challenge.

Table 1: Experimental design, measles neutralization titers, and measles viral titers in peripheral blood after challenge

	Rhesus Number	Sex	Vaccination Age (days)	Ween Age (days)	PRN titer day 42	Challenge Age (days)	MeVn copies (day 7 PC)[a]	TCID50 (day 7 PC)[a]
SC[b]								
	45294	F	28	183	2	244	6.6	1.7
	45338	M	18	199	56	234	4.3	0
	45406	F	26	193	1407	228	0	0
	45424	M	21	188	3647	223	0	0
MN [b]								
	45437	M	45	219	6800	261	0	0
	45478	F	32	206	3783	248	0	0
	45506	M	24	198	302	240	0	0
	45504	F	25	199	563	241	0	0
SC + MiG [b]								
	45537	M	49	216	8	258	7.1	3.4
	45546	F	42	209	87	251	7	2.5
	45555	M	36	203	75	245	6.8	3.2
	45559	F	34	201	2	243	6.7	3.3
MN + MiG [b]								
	45450	F	42	216	15	258	5.7	2.5
	45456	M	41	215	21	257	6.5	2.7
	45507	M	24	198	43	240	5.7	2.7
	45516	M	22	196	108	238	6.5	2.7
Unvaccinated [b]								
	44323	F	--	110		376	7.9	2.7
	44405	M	--	261		367	7.4	2.7
	44452	F	--	255		361	6.7	3
	44511	M	--	249		355	6.9	2.5

(PC) post measles virus challenge.

a. Copies of measles nucleoprotein RNA (MeVn) as log10 copies/10^6 PBMC or measles virus infectious titers as log10 $TCID_{50}/10^6$ PBMC

b. Rhesus macaques received subcutaneous (SC) or microneedle patch (MN) measles and rubella (MR) vaccination in the absence or presence of measles immune globulin (MiG) approximately 6 months prior to measles virus challenge.

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